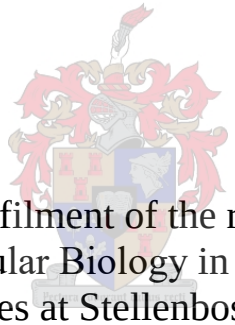
The influence of *Mycobacterium tuberculosis* on B cells during disease and treatment response.

by Willem Jacques du Plessis



Thesis presented in partial fulfilment of the requirements for the degree of Master of Science in Molecular Biology in the Faculty of Medicine and Health Sciences at Stellenbosch University

Supervisor: Professor Gerhard Walzl

Co-Supervisor: Doctor André G. Loxton

March 2016

Declaration

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

March 2016

Abstract

The therapeutic depletion therapy of B cells in humans have led to the discovery that B cells have the potential to function within immunity in a non-humoral fashion. Although this attribute wasn't fully appreciated, an ever growing body of evidence suggests otherwise. With *Mycobacterium tuberculosis* (*M.tb*), the causative agent of tuberculosis (TB), still endemic in many parts of the world it is crucial that previously under-appreciated cell types be studied for their potential participation in the disease to explore new avenues in curbing the disease.

Whole blood was collected from TB patients (n=52), other-lung disease controls (OLD, n=24) and healthy community controls (HHC, n=20) at diagnosis of disease, week 1 (after start of treatment) and week 24 (end of treatment, EOT). Antibodies to cell surface markers were utilised to stain whole blood for B cell immunophenotyping. In a separate experiment, B cells were enriched from peripheral blood mononuclear cells (PBMCs) isolated from interferon gamma release assay (IGRA) positive healthy community controls (n=11) and stimulated overnight with six antigens including phytohaemagglutinin (PHA), lipopolysaccharides (LPS), Bacillus Calmette–Guérin (BCG), Toll-like receptor 9 agonist (TLR9-a) and purified protein derivative (PPD). Following stimulation, B cells were assessed for functionality by multiplex analysis and *via* polychromatic flow cytometry.

Statistical analysis included non-parametric analysis with Mann-Whitney correction, one-way ANOVA and Wilcoxon rank sum tests with p-value adjustment done with the Bonferroni method. Comparisons were made between different groups (TB, OLD and HCC) and time points (diagnosis, week 1 and week 24) for the B cell immunophenotyping. Cytokine production was compared between the different stimulating conditions to assess functional capacity and we compared B cell phenotypes to identify the top contributing phenotype in terms of cytokine production. Our results revealed that memory-based B cells and marginal zone (MZ) B cells can distinguish between TB diagnosis and EOT, class-switched (CS) MZ B cells and non-class switched (NCS) mature B cells can distinguish TB from OLD. B cells readily produce cytokines in a stimulant dependent manner. BCG preferentially induce IL-1β. LPS and TLR9-a stimulation resulted in the highest cytokine-secretion upregulation. We also identified plasma-memory B cells ($CD19^{+}CD27^{+}CD138^{+}$) as the phenotype predominantly contributing to cytokine production.

Taken together these results not only indicate that TB disease has an influence on B cell frequencies, but that they also have the potential to function as effectors/regulators within the latent TB milieu. Future studies will investigate promising phenotypes as potential biomarkers and expand on B cell functional responses within the TB disease host-pathogen interaction.

Opsomming

Die sistematiese uitputtings terapie van B selle in mense het gelei na die ontdekking dat B selle die potensiaal het om te funksioneer binne immuniteit in ‘n nie-humorale manier. Alhoewel hierdie eienskappe nie ten volle waardeer was nie, is daar ‘n toenemende groei in die literatuur met bewyse wat andersins toon. Met *Mycobacterium tuberculosis* (*M.tb*), die veroorsakende agent vir tuberkulose (TB), wat steeds endemies is in verskeie dele van die wêreld is dit van uiterse belang dat sel tipes wat voorheen nie hoog op waarde geplaas was nie te ondersoek om hulle bydrae te evalueer in die poging om die siekte in toom te hou.

Heelbloed was versamel van TB pasiënte (n=52), ander-long siekte kontroles (OLD, n=24) en gesonde gemeenskap kontroles (HHC, n=20) by die diagnose van siekte, week 1 (na die begin van behandeling) en week 24 (einde van behandeling, EOT). Antiliggame teen sel oppervlak merkers was gebruik om heelbloed te vlek vir B sel immunofenotipering. In ‘n aparte eksperiment was B selle verryk vanaf perifere bloed mononukleêre limfosiete (PBMCs) wat verkry was van interferon gamma release assay (IGRA) positiewe gesonde gemeenskap kontroles (n=11), waarna dit oornag gestimuleer was met antigene wat onder andere phytohaemagglutinin (PHA), lipopolysaccharides (LPS), Bacillus Calmette–Guérin (BCG), Toll-like receptor 9 agonist (TLR9-a) and purified protein derivative (PPD) ingesluit het. Na die afloop van stimulasie was die B selle vir funksionele kapasiteit geëvalueer deur multiplex analise en *via* polichromatiese vloei sitometrie.

Statistiese analise het nie-parametriese analises met Mann-Whitney korreksie, een-rigting ANOVA en Wilcoxon rank sum test met p-waarde aanpassing deur die Bonferroni metode ingesluit. Vergelykings was gemaak tussen verskillende groepe (TB, OLD en HCC) en tussen verskillende tyd-punte (diagnose, week 1 en week 24) vir die B sel immunofenotipering. Sitokien produksie was vergelyk tussen verskillende stimulasie kondisies om funksionele kapasiteit te assesseer en ons het B sel fenotipes onderlangs vergelyk om die top sitokien produserende fenotipe te identifiseer. Ons resultate het getoon dat geheue-gebaseerde B selle en marginal zone (MZ) B selle onderskeid kan tref tussen TB diagnose en EOT, klas-omgeskakelde (CS) marginal zone B selle en nie-klas omgeskakelde (NCS) volwasse B selle onderskeid tussen TB en OLD kan tref. B selle produseer geredelik sitokiene in ‘n stimulant afhanklike wyse. BCG induseer IL-1β by voorkeur. LPS en TLR9-a stimulasie lewer die hoogste sitokien-afskeiding opregulasie. Ons het ook plasma-geheue B selle ($CD19^{+}CD27^{+}CD138^{+}$) geïdentifiseer as die fenotipe wat predominant bydrae tot sitokien produksie.

Als saam gevat toon hierdie resultate nie net dat TB siekte ‘n invloed op B sel frekwensies het nie, maar ook dat B selle die potensiaal het om te funksioneer as effektore/regulatore binne die TB milieu. Toekomstige studies sal belowende fenotipes ondersoek as potensiële biomerkers en uitbrei op funksionele B sel response binne die TB siekte gasheer-patogeen interaksie.

Aim

The aim of this project was to explore the influence of *M.tb* on B cells by identifying specific B cell phenotype frequencies and to assess specific functional capacity during TB disease and treatment response.

Objectives

To fulfil our aims we undertake the following:

Identify specific B cell phenotype – this will be done by assessing cell surface receptor expression by flow cytometry

Assess specific B cell functional capacity – this will be done by stimulating purified B cells and evaluating the intracellular as well as secreted cytokine profiles of the cells through Intracellular cytokine staining (Flow cytometry) and multi-cytokine analysis (MesoScale Discovery).

Hypothesis

We hypothesise that B cells play a role during *M.tb* infection and TB disease, and that treatment has an influence on its frequency, maturation and role as effectors/regulators.

Acknowledgements

I would like to thank the following people for their continued support and contribution towards the completion of my degree.

Firstly, I would like to express my sincere gratitude to my supervisors, Prof. Gerhard Walzl and especially to Dr. André Loxton, for the continuous support during the process of doing my master's degree for their patience, guidance, immense knowledge and experience that contributed to the successful completion of this study.

I would like to give a very special thank you to my family. Thank you for listening to me complain when the days were hard, thank you for encouraging me every step of the way and thank you for being there for me even when the reasons for me doing this aren't always clear to you. I wouldn't have been half the man I'm today without your ongoing support and guidance. I love you guys from the bottom of my heart.

My sincere thanks also goes to Dr. Nelita du Plessis, Dr. Léanie Kleynhans and Dr. Novel Chegou who either took time to train me in techniques, proof read my work or supported me to become the young scientist I am today.

A special thank you goes out to the SUN Immunology Research Group. From the nursing sisters (Shirley, Charmaine and Susan) who daily goes out to the clinics to gather samples all the way through to the research assistants who assisted me in samples. I can honestly say that it's a privilege, and inspirational, to have been part of a group that maintains such high standards across the board.

I would like to take the opportunity to thank all of my fellow lab mates, particularly Belinda Kriel, Sheena Ruzive and Lance Lucas for the stimulating discussions and for all of the fun we had together in the past three years which at times made seemingly impossible days bearable.

A big thank you goes out to Danni Ramduth and Nazma Mansoor of Becton Dickinson for their training, guidance and encouragement regarding all things flow cytometry during this project. I truly appreciate the time and effort you put aside to assist me with my technical queries.

Thank you to the National Research Foundation for the financial support given to me during the duration of this project. It has truly made this endeavour a possibility for me and I hope the knowledge gained through this project benefits the foundation.

Table of contents

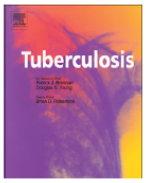
Chapter 1:

B cells as multi-functional players during Mycobacterium tuberculosis infection and disease

Contents lists available at ScienceDirect

Tuberculosis

journal homepage: http://intl.elsevierhealth.com/journals/tube



REVIEW

B cells as multi-functional players during *Mycobacterium tuberculosis* infection and disease

Willem J. du Plessis, Gerhard Walzl, André G. Loxton*

SA MRC Centre for TB Research, DST/NRF Centre of Excellence for Biomedical Tuberculosis Research, Division of Molecular Biology and Human Genetics, Faculty of Medicine and Health Sciences, Stellenbosch University, PO Box 241, Cape Town 8000, South Africa

ARTICLE INFO

Article history:
Received 18 June 2015
Received in revised form
28 September 2015
Accepted 15 October 2015

Keywords:
B cell
Tuberculosis
HIV/TB co-infection
Immune activation
Cytokine

SUMMARY

Immunity to tuberculosis is still understood to be driven and maintained by T-cell derived immune responses. With a steady influx of data, it is becoming clear that B cells, the mediators of humoral immunity, have the capacity to function in roles not previously appreciated within the traditional B cell dogma. In this review we aim to discuss B cells, from its generation through to its functioning as effectors in both the innate and adaptive immune response, within the tuberculosis domain.

The work presented in this chapter has been formatted in the style of Tuberculosis journal to which it was submitted and accepted for publication.

B cells as multi-functional players during *Mycobacterium tuberculosis* infection and disease.

Willem J du Plessis[a], Gerhard Walzl[a] and André G Loxton[a]

[a] SA MRC Centre for TB Research, DST/NRF Centre of Excellence for Biomedical Tuberculosis Research, Division of Molecular Biology and Human Genetics, Faculty of Medicine and Health Sciences, Stellenbosch University, Cape Town, South Africa

PO Box 241 Cape Town, 8000 South Africa

Corresponding Author:

Dr AG Loxton

Email: GL2@sun.ac.za

Tel: (+27)-21 9389399

Fax: (+27) 86 614 0216

Abstract

Immunity to tuberculosis is still understood to be driven and maintained by T-cell derived immune responses. With a steady influx of data, it is becoming clear that B cells, the mediators of humoral immunity, have the capacity to function in roles not previously appreciated within the traditional B cell dogma. In this review we aim to discuss B cells, from its generation through to its functioning as effectors in both the innate and adaptive immune response, within the tuberculosis domain.

Keywords

B cell, Tuberculosis, HIV/TB Co-Infection, Immune Activation, Cytokine

Introduction

Mycobacterium tuberculosis (*M.tb*) is the causative agent of tuberculosis (TB) and was responsible for 9 million newly reported TB cases during 2013 [1]. Although the World Health Organisation (WHO) reported that TB mortality has fallen by 41% since 1990 [2], the organism was still responsible for claiming 1.5 million lives during 2013 [1]. More than a century [3] of dedicated research into abolishing this threat has passed and the disease is still regarded as the most successful known infectious pathogen to man. During the 1990's, TB was declared a global emergency by the WHO [4], launching the "Framework for effective TB control" initiative that defined five required elements in controlling TB [5]. These globally accepted five elements are: (i) a government that is committed to the maintainable control of tuberculosis; (ii) using sputum-smear for diagnosis, mainly among patients who self-refer to health services with symptoms; (iii) utilising standardized short-course chemotherapy coupled with appropriate management conditions, including direct observation treatment (DOT); (iv) a functioning drug supply system; and lastly, (v) a recording and reporting system that allows the assessment of treatment outcome [4]. In 1995 this package was branded under the name Directly Observed Therapy, Short-course (DOTS) and subsequently became one of the most well-known brands in health. It is estimated that 4.6 – 6.3 million lives were saved between the programme's inception in 1995 and 2009 from the 49 million tuberculosis patients treated under the DOTS/Stop TB Strategy during this time frame [6]. Despite the fact that current TB management strategies is proving to be successful with increased patient cure and ultimately saving lives, the resulting impact on the epidemiology has shown to be less than expected [7]. Risk factors associated with an individual's susceptibility to TB, on a population level, include poor working and living conditions that facilitate TB transmission, and include factors like HIV infection, malnutrition, diabetes, alcohol abuse and indoor air pollution [7]. It is furthermore estimated that two billion people live with latent *M.tb* infection, where these people may serve as a reservoir for future active disease cases [8]. Latent tuberculosis infection (LTBI) is defined as being infected with the causative agent, but lacking clinical symptoms, without being exempt of future development into clinical disease. *M.tb* interacts with the host immune system in a very complex fashion. Not all people who are infected progress to active disease; latently infected individuals have a 10% lifetime risk of developing active disease with clinical symptoms. Immunosuppressive triggers such as anti-tumour necrosis factor (TNF) therapy for unrelated diseases, HIV infection and diabetes greatly enhances this risk, but it can also be reduced with prolonged isoniazid prophylaxis [9]. Conclusions made from studies specifically focussed on latent infection postulates that LTBI (latent tuberculosis infection) and active tuberculosis disease aren't mutually exclusive but rather represent a spectrum of disease states that is dynamically influenced by the host- and pathogen's interactions [10]. Being able to accurately distinguish between latent and active phases of the tuberculosis disease would be of supreme benefit to the proper management and eradication of the epidemic. The term "biomarker" is a portmanteau of "biological marker" and by definition refers to a broad sub-classification of medical signs (outside observations from the patient that objectively quantifies its medical state) which can be measured in an accurate and reproducible fashion [11]. An encompassing review on immunological biomarkers in tuberculosis by Walzl *et al*. defined the ideal TB biomarker as being capable of: (i) distinguishing between active and latent tuberculosis

disease in patients, (ii) returning to basal levels during treatment, (iii) reproducibly predicting clinical outcomes (cure, relapse or sterilizing cure of *M. tuberculosis* infection) in diverse population settings and finally, (iv) predicting the efficacy of vaccines and serve as end points in clinical trials [12]. With MDR-TB (multi-drug resistant tuberculosis) and XDR-TB (extensive-drug resistant tuberculosis) becoming more prevalent, the need for effective biomarkers is paramount. Recent studies have broadened the views of biomarker research to not only focus on host cytokine responses [13–16], but also micro-RNA (miRNA) [17–19] and to even diagnose tuberculosis on a serological basis by combining different immunoglobulin (Ig) classes against selected *M.tb* targets [20]. This raises the possibility of the validity of utilising B cells as biomarkers in tuberculosis disease, not only by measuring their capacity as effectors/regulators in expressing cytokines, but also on a phenotypic level and observing dynamic changes in population groups.

Lymphocytes subsets (T, B and natural killer (NK) cells) have similar appearances under the microscope, but can clearly be distinguished by their function. One of the characteristics which differentiates lymphocytes from other immune cells is the unique antigen receptors they contain following a recombination process of the V, D and J gene segments. For B cells this results in functional IgH and L-chain genes. The T cell counterpart results in the T cell receptor (TCR) gene repertoire, including TCR α, β, δ and γ [21]. This empowers the immune system to have very high specificity to a broad spectrum of antigenic challenges. Some of the functions associated with lymphocytes are the production of antibodies, the regulation and drive of the immune system and direct cell-mediated killing of tumour cells and cells that are intracellularly infected with pathogens [22]. Classically, B cells are known for producing antibodies which are the primary effectors of humoral immunity. These antibodies have the ability to nullify elements harmful to the body, including extracellular pathogens and toxins. Other functions of B cells include the activation of the compliment system and to facilitate opsonisation.

Characteristically, tuberculosis research was T-cell driven because of the pathogens intracellular nature. And although numerous advances in delineating tuberculosis disease has been made, it still remains an unsolved problem and accounts for great loss and morbidity globally. It is now clear that previously under-appreciated cell types are playing a prominent role in the immune response to tuberculosis. This review aims to give an overview of B cells during disease (HIV-TB coinfection), B cell development, its role during immune activation as well as the functional capacity of effector/memory B cells and their regulatory role.

The TB/HIV syndemic

Human immunodeficiency virus (HIV) primarily infects lung lymphocytes and, like tuberculosis, alveolar macrophages. This accounts for the poor prognosis concerning patients with HIV and TB co-infection. HIV actively reduces the host's specific immune defence by infecting all major lymphocyte populations (including B cells) while TB flourishes in compromised immune environments. It is now well established that HIV rigorously impairs lung immune responses [23].

Elementary work done by Young *et al.* in 1985 compared Broncho alveolar lavage (BAL) immunoglobulins from HIV infected patients displaying pulmonary symptoms with uninfected individuals and found increased levels of total IgG, IgM and IgA [24]. This supports the notion that HIV infection results in the systematic polyclonal activation of B cells.

Acute infection with HIV-1 results in a detrimental delay and decrease in the host's humoral immune response, rendering it ineffective. Integrin $\alpha_4\beta_7$ on T cells are the binding site for the envelope protein gp120 of HIV-1 virus. The virus also uses this protein to signal through to the cell. Jelicic *et al.* recently showed that gp120 also binds naïve B cells through $\alpha_4\beta_7$ and that this resulted in an abortive proliferative response. When applied to primary B cells, signalling through this complex resulted in increased expression of both TGF-β1 and FcRL4, whose functions are as an immunosuppressive cytokine and inhibitory B cell receptor respectively. The group further showed that co-culturing of autologous $CD4^+$ T cells (infected with HIV-1) with B cells resulted in an even higher increase of FcRL4 expression on B cells [25]. This work showed that viral proteins not only facilitates the chronic activation of the immune system, but that they play a central role in HIV-1 associated B cell dysfunction. The work identified a potential mechanism the virus employs to subvert early humoral responses during infection. The early role of B cells in the activation of T cells, can thus directly affect the host's immune response to T cell driven protection in diseases like tuberculosis.

A recent study showed that natural autoantibodies found in human serum have the capability to be functional in the protection against in vitro HIV-1 infection [26]. These autoantibodies were denoted as IgG-reactive antibodies, and an immunoglobulin subclass analysis showed that IgG2 were dominant in presence, both in Gamma Bind G Sepharose Flow through (GBF) and normal human serum (NHS). Isolated IgG-reactive antibodies from the GBF fraction neutralized an in vitro HIV-1BaL strain infection with almost 100% effectiveness when a 2µg/ml concentration was used [26]. These findings support the notion that IgG reactive antibodies should be investigated as a possible use in the treatment of HIV-1.

Plasmablasts are the result of B cells that have undergone terminal differentiation following infection or vaccination. These cells then circulate transiently in the blood, with the ability to produce antibody. But, as a hallmark of HIV, B cells surmise to hyperactivity and undergo this terminal form of differentiation early, and abnormally high levels of these plasmablasts are observed in viremic individuals [27].

Buckner *et al.* reported that HIV viremic individuals display increased amounts of IgG^+ plasmablasts not only in the early phases of disease, but predominantly in individuals with chronic HIV viremia as opposed to the predominant IgA^+ plasmablast profile observed in both HIV-negative and aviral HIV-infected individuals who are on treatment [27]. This study also concluded that although plasmablasts may contribute specifically to the HIV immune response, that this B cell response is not HIV specific and arise prematurely on the suggested basis of indirect immune-activation effects of HIV replication [27]. This level of B cell dysregulation might explain why HIV infected individuals have insufficient antibody responses, even during early phases of the infection.

The immunological environment of HIV/TB co-infection is not only characterised by this incomplete humoral response, but also by cytokine and chemokine irregularities that results in immune activation, increased viral replication and T cell dysfunction [28–32]. TB treatment significantly improved the production of Th1 cytokines and chemokines when compared between HIV positive and HIV negative tuberculosis patients [33].

B cells: from bone marrow to plasma cells

There are three subsets into which naïve B cells can be categorised, namely: follicular B cells, marginal zone (MZ) B cells and B-1 B cells. The B-1 B cells can be partitioned further into B-1a and B-1b B cells.

Two functional domains are occupied by recirculating mature, but naïve follicular B cells. As soon as follicular B cells mature and gain the ability to recirculate, they start migrating repeatedly through the lymph and blood to areas occupied by B cells in Peyer's patches, the spleen and lymph nodes. The follicular niche of B cells represents the primary location whereby recirculating B cells mediate T-dependent immune responses to protein antigens, as naïve B cells residing in this well-established niche have the ability to present T-dependent antigens to activated T cells [34]. T-dependent B cell activation by TLR ligands on B cells is a topic of debate [35,36], however, it is probable that synergistic signals (mediated through the B cell-receptor (BCR), TLR and CD40) are received by B cells activated in the follicular niche by T-cell dependent antigens of microbial origin.

B cells originate from B cell precursors. Before birth, the liver serves as a source of some B cell progenitors whereas post-natally the bone marrow is the primary source for the production of B cells. When cells are between the developmental stages of immature bone marrow (BM) B lineage cells and matured naïve B cells, found in secondary lymphoid tissues and peripheral blood, they are known as 'transitional B cells' [37]. Transitional B cells undergo a selection process to eliminate cells that test positive for auto-reactivity. The cells that survive this selection process continue to develop into naïve B cells [38–40]. These cells can further be classified into two distinct proposed subsets, duly known as stage 1 (T1) and stage 2 (T2). A recent immunophenotyping study identified the two subsets in normal bone marrow, with T1 showing a phenotype of $CD45^{+}CD19^{+}CD10^{+}IgM^{+}IgD^{lo}$ and T2 with a phenotype of $CD45^{+}CD19^{+}CD10^{+}IgM^{+}IgD^{+}$ with frequencies of 3.2- and 3.1% respectively [37]. It is also believed that transitional B cells have the ability to differentiate from T1 to T2 cells within the bone marrow.

Vossenkämper *et al.* [41] performed an interesting study where they showed that a major component of immature B cells (in the transitional 2, or T2 phase) emigrate to the gut associated lymphoid tissue (GALT) where they become activated. The activation process from immature B cells to mature naïve B cells in the GALT is a previously unknown mechanism for these cells. Autoreactive cells are removed from the developing repertoire during maturation, a process which fails in individuals diagnosed with systemic lupus erythematosus (SLE). Using SLE as a model, Vossenkämper *et al.* demonstrated that immature B cells in SLE individuals not only struggle to access the gut, but also that the gut immune compartments are depleted during this disease state. It is therefore suggested that the activation of immature B cells in the GALT function as a control point

against autoimmunity and, in healthy individuals, serve as a venue for a vast populations of IgA plasma cells and marginal zone B cells [41].

B cells are the only cells with the capacity to produce antibodies. They undergo vast development in their cytoplasm that is coupled with the accumulation of organelles associated in protein expression, like the polyribosomes and endoplasmic reticulum [42]. The genesis of these unique ultrastructures empowers B cells to synthesize proteins. Plasma cells represent between 0.1–1% of cells in lymphoid organs, but they have the capacity to produce thousands of antibody molecules per second that prove to be adequate in maintaining global antibody levels [42–44], highlighting their lack of other immune functions because of the extreme specialization. Although it is known that B cells produce cytokines [45–49], little is known about the phenotypic features that characterize cytokine producing B cells. Being able to identify the phenotypic features that could distinguish between proinflammatory and anti-inflammatory B cell subsets that produce cytokine would be of great significance in the clinic as novel approaches in targeting specific plasma populations involved in the production of pathogenic autoantibodies can be removed without diminishing the effect of IL-10 producing B cells that confer protection during autoimmune disease.

The role of B cells in immune activation

Peripheral B cells play a role in mediating humoral immune responses, but they have shown to also produce cytokine and function as antigen presenting cells for naïve T cells. Less well understood B cell functions include a repertoire of specific B cells that act as regulatory cells with the ability to inhibit tissue-specific inflammation [50], the manner in which T cells (involved in immune drive) are activated by B cell derived cytokine [51], and finally their influence in the initiation of tertiary lymphoid organs at disease-related sites of inflammation [52].

Marginal zone B cells are named as such because of their location. The spleens' marginal zone synoids hosts these cells where they excel at responding to antigenic exposure [53]. This is an ideal location for the cells to be housed as these synoids are the location where blood born antigens are filtered through. This gives the B cells the opportunity to function as antigen presenting cells (APCs).

One area of uncertainty is the influence that antibodies (Abs) have during TB infection. Essentially, FCγR modulate humoral immunity by engaging immunoglobulins (Igs) produced by B cells. During tuberculosis challenge, the engagement of FCγRIIB could result in a diminished TH1 response by attenuating IL-12p40 or the activation of antigen presenting cells (APCs) [54]. Furthermore, C57BL/6 mice that lacked the γ-chain (shared with the activation of FCγR) had greater susceptibility and immunopathology after *M.tb* challenge, associated with elevated IL-10 levels [54]. This demonstrates that the activation of distinct FCγR can affect the produced cytokine profile and thus, the susceptibility to *M.tb* infection. A recent study identified a FCγ gene (FCGR1A – high-affinity IgG FC receptor 1) with significantly higher expression in active tuberculosis patients as compared to participants with LTBI before treatment, irrespective of HIV status or genetic

background [55], identifying it as a possible biomarker for the classification of active tuberculosis. Taken together, these reports warrant further FCγ research to fully define their role and function during tuberculosis.

Mature B cells express the transmembrane activator and CAML interactor (TACI), which is part of the tumour necrosis factor (TNF) receptor (TNFR) family. B cell activation factor (BAFF), which is a TNF homologue, is a TACI ligand. BAFF plays a fundamental role in the survival of both transitional and mature B cells and is expressed on monocytes, macrophages and dendritic cells (DCs) [56–58]. This role became apparent when BAFF-deficient mice demonstrated an almost complete halt in T1 and T2 stage B cell development in the spleen, with subsequent loss of MZ and follicular B cells but B1 B cells remaining intact [59–61]. More recently, Liu and colleagues [62] demonstrated that during active tuberculosis disease, the mRNA and protein expression levels of BAFF and APRIL (a proliferation inducing ligand) were significantly up-regulated. Furthermore, the distinctive increase of these proteins in patients with tuberculosis pleurisy may prove them valuable as biomarkers for indicating the patients' current disease status [62].

Functional B cells – their influence as effectors and regulators of immunity

The regulation of immune responses is a complex and interesting field of research which has seen a lot of growth in the past 20 years. A growing theory in the field of immune regulation is that it's the cooperation of host of immune cells with both effector and regulatory functions that contribute to immune homeostasis. Evidence shows that a combination of innate and adaptive immune recognition mechanisms collaborate for the optimal recruitment of various cell types that interact to ultimately produce effector and regulatory functions. Immune homeostasis is all about the balance that needs to be achieved between tolerance versus self and harmless antigens and the governing of immune amplification responses during foreign antigenic challenge. The term "immunological tolerance" was first coined in the tissue transplantation community [63] and can be described as a non-reactive state towards a substance that would otherwise be expected to elicit an immunological response. The difference between tolerance and control is that tolerance inhibits lymphocyte activation whereas control governs the activity of effector cells. Although a whole new wave of research focussing on B cells as regulators of immunity is observed, evidence for their role in immune suppression dates back to the 1970's [64,65]. Nonetheless, as will be discussed further in this review, various studies have identified the prominent role B cells play during immune-driven inflammation and control.

The activation of B cells can lead to a range of outcomes to the host, either by producing antibody, secreting cytokines [including interleukin (IL)-4, IL-6, IL-10, IL-12 and interferon(IFN)-gamma] or presenting antigen to naïve T cells [49,66–68]. In short, B cell responses are beneficial to the host during infections and damaging during autoimmune disease. It should be mentioned however, that B cells have the capacity to limit the hosts defence against pathogens and shield against autoimmune pathologies. This demonstrates that B cells can have distinct roles as drivers and regulators of immunity depending on the functional properties they gain following activation. For example, the resolution of experimental autoimmune encephalomyelitis (EAE) is regulated by

B cell-produced IL-10 [48]. This regulatory function mediated by IL-10 was also shown in models of arthritis and ulcerative colitis [69,70]. B cell produced IL-10 provide a general mechanism of immune protection as this is demonstrated in a study by Neves *et al.* where IL-10 inhibited immune protection against *Salmonella typhimurium* in mice [68]. An IL-10 reporter mouse strain was generated by inserting the sequence coding for enhanced Green Fluorescent Protein (eGFP) into the *IL-10* locus, where after it was utilised to demonstrate IL-10 producing B cells within 24 hours after challenge in the spleen, while these B cells were absent in naïve mouse [68]. This shows that IL-10 expressing B cells develop as a prompt response before the formation of the extra-follicular plasma cell population (typically developing around 3 days after immune stimulation) and the germinal centres, which generally appear 9-12 days following challenge. Upon further inspection, flow cytometric analysis revealed on a single cell level that all of the IL-10 producing B cells expressed the CD138 receptor, which is a hallmark marker for plasma cells [68]. Collectively, the study demonstrated that a plasma cell subset rapidly responded to antigenic challenge by up-regulating IL-10 expression and subsequently mitigating the protective anti-microbial innate response. Therefore, immunity can be regulated by cytokines produced from plasma cells, suggesting the existence of regulatory plasma cells (Preg).

Toll-like receptor (TLR) agonists have demonstrated to be instrumental in IL-10 secretion by naïve B cells *in vitro* and that TLR controlled regulatory B cell functions *in vivo* [71,72]. Work from our group suggest that TLR9 is a potent stimulant of IL-10, IL-6 and TNF-α during latent TB infection (unpublished). It can subsequently be deduced that the inhibitory functions of B cells form part of a counter-regulatory loop that is endorsed directly by the signals stimulating immunity [73].

The five isotypes of antibody that B cells produce are immunoglobulin (Ig)-A, IgD, IgG, IgE and IgM. Of these five IgA is the most abundant in the body and constitutes more than 70% at steady state [74]. The host gut contains roughly 10^{12} bacteria per gram of luminal content and the high number of IgA plays a crucial role in maintenance of the interplay between the host and the commensal microbiota [74,75]. The production of IgA is vitally influenced by both tumour necrosis factor (TNF)-alpha and iNOS, the inducible nitric oxide synthase [76,77]. Fritz *et al.* did an independent characterization of cells in the gut that express these two factors and demonstrated that plasma cells contributed largely to the production of both iNOS and TNFα in the small intestine lamina propria [77]. This was confirmed when reduced IgA titres and number of IgA plasma cells were observed in the lamina propria of mice with a B cell restricted deficiency in iNOS and TNFα [77]. These data would suggest that plasma cells, by producing soluble mediators, can in fact modulate immunoglobulin isotype switching [78]. It is also possible that this effect operates by an autocrine loop, giving plasma cells the freedom to regulate their own process of isotype switching, or otherwise involve on an instructive effect other neighbouring plasma cells to switch isotype [78]. Maaser *et al.* infected mice with *Citrobacter rodentium*, a murine model pathogen for human enteropathogenic *E. coli*, and showed that successful clearance of the infection was dependent on the presence of B cells and not secretory IgA or IgM antibodies [79]. Taken together, these data demonstrate that the regulation of humoral immunity and the communication between the host and its commensal bacteria can be mediated by gut plasma cells in an antibody-independent fashion that involves soluble factors.

An investigation by Bermejo *et al.* revealed that $CD19^{+}CD138^{+}$ plasma cells were the primary producers of IL-17 during peak *Trypanosoma cruzi* infection in the spleen [80]. At the peak of the response, which was 10 days' post challenge, IL-17 was expressed by approximately 10% of the $CD19^{+}CD138^{+}$ population. The impact on disease outcome by B cell-produced IL-17 was assessed by reconstituting mice devoid of B cells with either IL-17-deficient- or wild-type B cells. The results demonstrated that mice with B cell related IL-17-deficiencies had increased TNF-α and IFN-γ production coupled with intensified immunopathology and hastened death when compared to controls [80]. When live *Trypanosoma cruzi* trypomastigotes were added *in vitro* to the culture, IL-17 expression in B cells were instigated by a parasite dose-dependent manner. They continued to show that this process was self-sufficient from cytokines, such as IL-6 and IL-23, and transcription factors including RORγt, Ahr and RORα, which are required for the production of IL-17 by T cells or other innate lymphocytes. Further independence from key B cell activation pathways, such as MyD88 and CD40 were established [80]. These results prove that B cells can be activated by pathways that are TLR-independent. Here, it was shown that B cell activation was mediated *via* a mechanism that modifies the glycosylation of B cell surface molecules through a parasite derived trans-sialidase [80].

Seeing that B cells have the ability to produce cytokines following polyclonal activation through TLR, which responds to conserved molecules on pathogens collectively known as pathogen-associated molecular patterns (PAMPS), it is suggested that B cells participate in sepsis. Sepsis is the result of widespread infections of Gram-positive and Gram-negative bacteria or fungi [81] and is characterised by an initial stage of overpowering inflammation which can progress to septic shock and death if left uncheck [82]. The subsequent phase is defined by prolonged immune suppression, which proves to have the most fatalities as patients are left highly susceptible to nosocomial infections [83]. Kelly-Scumpia *et al.* identified a novel role for B cells during early innate responses during sepsis [84]. They showed that Rag1 (-/-) mice lacked sufficient early inflammatory responses and displayed reduced longevity during sepsis [84]. Decreased chemokine and inflammatory cytokine production in anti-CD20 B cell-depleted and B cell deficient mice (but interestingly not in α/β T cell-deficient mice) coupled with reduced survival post sepsis was observed [84]. They continued to show that a type I interferon (IFN-I) receptor is responsible for the activation of both marginal zone and follicular B cells, ultimately identifying the protective role during sepsis to be mediated by IFN-I-activated B cells during early innate responses to sepsis [84].

Further evidence for the early role that B cells play during immunological responses was demonstrated by Rauch and colleagues [85] when they identified an effector B cell subset, duly described as innate response activator (IRA)-B cells – in the light of granulocyte-macrophage colony stimulating factor's (GM-CSF) known role in the activation of innate leukocytes – as the primary producers of GM-CSF in the spleen during experimental sepsis. This finding was surprising, as it's believed that GM-CSF is mainly produced *in vivo* by non-hematopoietic cells, macrophages and in some cases, T cells [86,87] [88]. Nonetheless, GM-CSF expressing B cells accounted for roughly 75% of GM-CSF expressing cells in the spleen, under the stimulating conditions which utilised lipopolysaccharide (LPS) [85]. This response required intrinsic signalling *via* TLR4 and MyD88 [85]. This data is in line with the concept that microbial products directly trigger the TLR4 and

MyD88 responses in B cells. Remarkably, flow cytometric analysis implicated IgM^+CD138^+ B cells as the producers of GM-CSF, who also demonstrated to secrete IgM^+ antibody, proving that they are indeed plasma cells [85]. When compared to the controls, mice lacking B cell derived GM-CSF succumbed to death sooner, which correlated with increased neutrophilia, bacterial burden, and elevated levels of the pro-inflammatory cytokines IL-1β, TNF-α and IL-6 [85]. GM-CSF is a pleiotropic cytokine which influences its targets cell's production, maturation, function and survival. Thus, this indicates that plasma B cells may play a key role in the control of disseminated microbial infection by producing GM-CSF and subsequently limiting the severity of pathogenic inflammation. IRA-B cells stem from B1a B cells, are functionally and phenotypically distinct, relies on pattern recognition receptors and have the capacity to produce GM-CSF [85]. A recent study by Weber *et al*. continued to show the influence of IRA-B cells as early responders to bacterial infection through the production of GM-CSF, resulting in a previously unrecognised GM-CSF-IgM axis [89]. In response to lung infection (a model for pneumonia disease was utilised), IgM was secreted by B1a B cells after they have migrated from the pleural space into the lung parenchyma, in an IRA-B cell dependent fashion [89]. Effective early defensive responses are ensured in the lungs by the strategic location of IRA-B cells coupled with their capacity to produce GM-CSF-dependent IgM confirming B cells as orchestrators of protective IgM immunity [89].

As antigen presenting cells, B cells play a role in the proper activation of T cells, and thus direct $CD4^+$ T-cell responses. A recent study into the effects of human B cells on T cell plasticity challenged the common notion that $CD4^+$ T cells follows a strict fate commitment during differentiation into distinct subsets. Human B cells were responsible for the induction of prominent and stable co-expression of T_H1 and follicular helper T (T_{FH}) cell functions during antigenic presentation and recall [90]. The study, which followed $CD4^+$ T cell differentiation by primary human B cells, used tetanus toxoid and Salmonella species as antigen models to demonstrate that T_H1/ T_{FH} both express the effector cytokines IFN-γ and IL-21 [90]. The expression of IL-21 and IFN-γ was respectively controlled by B cell produced IL-6 and IL-12, where IL-21 proved to be instrumental for humoral immunity. The study concluded that human B cells exploit the plasticity of $CD4^+$ T cells to facilitate flexibility within the effector T cell response. It is postulated that B cells induce a T cell subset to co-express IL-21 and IFN-γ, for the dual function of T cells mediating antibody production *via* IL-21 whilst still maintaining T_H1 cytokine expression through IFN-γ to support other cellular immune defences [90]. This raises the possibility of an immune-evasion mechanism for tuberculosis, where B cells might be affected in some way that proper T cell activation does not occur, resulting in a lesser immune response, ultimately favouring the bacterial infection.

B cells go through a broad spectrum of differentiation during its developmental stages. The currently known B cell repertoire includes, in order of the first progenitor to terminally differentiated cells: pluripotent hematopoietic stem cells (pHSC), common lymphoid progenitor (CLP), pro-B cells, pre-B cells, immature B cells, mature B cells, marginal zone B cells, memory B cells and finally, plasma cells. It's because of this diversity seen in B cell differentiation that multiple effector functions are associated with B cells, depending on the developmental status of the recruited B cell and the antigenic stimuli. But not all B cells are dependent

on antigenic stimulation. Specialized subsets of B cells, namely B1 ($CD5^+$) B cells and marginal zone (MZ) B cells readily respond to T cell independent antigens in a manner that suggests the existence of innate like B cell memory [53]. Another function of BAFF is to regulate T cell-independent (TI) IgM production. Jones *et al.* showed that during an *Ehrlichia muris* infection BAFF was required for TI IgM secretion in the spleen [91]. Their findings concluded that the TI host defense can be modulated by BAFF signalling, through influencing the late stage differentiation of B cells where it regulates plasmablast differentiation and/or IgM secretion. B cells are playing an ever increasing important role in the general regulation of host defense, especially so in disease states that is predominantly regulated via a T cell dominant response. B cells are required for the proper activation of T cells. It is becoming more apparent how important it is to maintain the healthy collaboration between these two cell types. It is known, for example, that the dysregulation of T cell survival and apoptosis is a common cause of autoimmune diseases, like multiple sclerosis (MS). Xiao and colleagues showed that high levels of BAFF was associated with increased T cell tenacity against apoptosis [92]. Their mouse model demonstrated that BAFF up-regulated osteopontin (OPN) secretion from B cells (during autoimmune disease) which subsequently lead to the expression of Bcl2 on T cells, which is an anti-apoptotic molecule.

Maglione *et al.* demonstrated that the presence of B cells contributed to host protection of mice infected with pulmonary tuberculosis. They showed that the presence of CXCL13 (a B cell chemoattractant) was negatively regulated by the presence of B cells, and that the absence of B cells (in B $cell^{-/-}$ mice) resulted in intensified immunopathology that was linked to excessive recruitment of neutrophils to the lungs. Interestingly, infected B $cell^{-/-}$ mice had increased pulmonary production of the anti-inflammatory cytokine IL-10, whereas the production of pro-inflammatory cytokines IFN-γ and TNF-α remained unchanged from the wild type. The protective role of B cells in the immune response was confirmed with the adoptive transfer of these cells to B $cell^{-/-}$ mice, where they complimented the phenotype [93].

B cell miRNA

MicroRNAs (miRNAs) are small RNA molecules that play a key role in host gene expression regulation. Although these molecules only consist of sequences ranging between 20 – 23 nucleotides in length, their role in gene regulation on a post transcriptional level has been made clear. Their modus operandi is to use the RNA interference pathway to regulate mRNA translation. These endogenous, single stranded RNA molecules are the result of processing full length mRNA transcripts [94]. The ribonucleoprotein complex RISC (RNA-induced silencing complex) that facilitates post-transcriptional gene silencing consists of mature miRNAs and Argonaute (Ago) proteins [95]. The process of regulation starts with a complimentary base pairing of the miRNA where after it guides RISC to the target mRNAs. After this, the mRNAs are degraded, destabilized or translationally inhibited by the Ago protein [96,97]. To date more than 1500 miRNAs have been identified [98], where they play various cell regulatory roles including development [99,100] and pathogenesis [101,102].

With the advances made to the description and role of miRNAs involved in major biological processes, a database was created with the aim of providing an integrated platform with comprehensive miRNA sequence data, annotation and gene predicted targets. The database (miRBase) fulfils three major roles: the registry firstly functions as an independent arbiter of miRNA gene nomenclature, secondly it is the primary online repository for miRNA sequence data and annotation and thirdly, it provides a comprehensive database for the prediction of miRNA gene targets [103,104]. miRBase is freely available at http://microrna.sanger.ac.uk

miRNA involvement in the development of B cell lymphogenesis and lymphomagenesis is largely unknown.

Jensen *et al.* has recently shown that the miRNA profile for precursor B cells stayed the same regardless of aging when comparing children with adults. But, that it was rather much more linked to the specific stage the B cell is in. Importantly though, it was noted that specific age-dependant differences was observed that are involved in key networks, including cell differentiation, which could be associated with the altered production potential of cell subsets coupled with age [105].

B cell depletion therapy in clinical trials have resulted in the substantial reduction in new relapses in patients with multiple sclerosis (MS), implicating them in the disease's pathophysiology. One of the proposed mechanisms by which B cells contribute to the exacerbation of the disease is by the over-activation of T cells *via* irregular secretion of B cell pro-inflammatory cytokines. To elucidate the mechanism by which this dysregulated B cell cytokine expression in MS occurs, Miyazaki and colleagues screened candidate miRNA in activated B cells of MS patients [106]. Their findings demonstrated a correlation between abnormal increased secretions of TNF-α and lymphotoxin (LT) to the abnormal expression of miR-132. This observation was confirmed when the over-expression of miR-132 resulted in the significant increase of TNF-α and LT by MS B cells [106]. The target of miR-132, sirtuin-1, was also suppressed during the overexpression of miR-132. The pharmacological inhibition of sirtuin-1 in normal B cells resulted in the aberrant expression of TNF-α and LT, whilst the abnormal production of these cytokines were reversed by the addition of resveratrol, a sirtuin-1 activator. Ultimately, these results defined a novel miR-132-sirtuin-1 axis that regulates pro-inflammatory cytokine production by human B cells and whose dysregulation results in pathophysiology as seen in MS [106].

Conclusion and future perspectives?

B cells provide a familiar yet innovative avenue for investigation during TB disease. Future studies into the function of these cells and subsets during infectious diseases, like tuberculosis, should aim to address the exact phenotype and functional potential under conditions of treatment, failure and relapse. These findings should be coupled to B cell derived gene expression signatures and miRNA (as potential biomarker) as targets for the treatment and prevention of relapse/recurrence of disease.

Acknowledgements

The financial assistance of the National Research Foundation (NRF) towards the research project of WJ du Plessis is hereby acknowledged. Opinions expressed and conclusions arrived at, are those of the authors and are not necessarily to be attributed to the NRF. GW holds the South African Research Chair for Biomarkers in Tuberculosis grant.

References

[1] WHO. Global Tuberculosis Report 2014. World Heal Organ 2014. http://www.who.int/tb/publications/gloabl_report/en/.

[2] World Health Organisation. Global Tuberculosis Report 2012 2012:89. http://www.who.int/tb/publications/global_report/en/.

[3] Daniel TM. The history of tuberculosis. Respir Med 2006;100:1862–70. doi:10.1016/j.rmed.2006.08.006.

[4] Raviglione MC. The TB epidemic from 1992 to 2002. Tuberculosis (Edinb) 2003;83:4–14. doi:10.1016/S1472-9792(02)00071-9.

[5] World Health Organization (WHO). World Health Organization Global Tuberculosis Programme - Framework For Effective Tuberculosis Control. Doc WHO/TB/94179 n.d. http://www.who.int/tb/publications/1994/en/.

[6] Glaziou P, Floyd K, Korenromp EL, Sismanidis C, Bierrenbach AL, Williams BG, Atun R, Raviglione M. Lives saved by tuberculosis control and prospects for achieving the 2015 global target for reducing tuberculosis mortality. Bull World Health Organ 2011;89:573–82. doi:10.2471/BLT.11.087510.

[7] Lönnroth K, Jaramillo E, Williams BG, Dye C, Raviglione M. Drivers of tuberculosis epidemics: the role of risk factors and social determinants. Soc Sci Med 2009;68:2240–6. doi:10.1016/j.socscimed.2009.03.041.

[8] Corbett EL, Watt CJ, Walker N, Maher D, Williams BG, Raviglione MC, Dye C. The growing burden of tuberculosis: global trends and interactions with the HIV epidemic. Arch Intern Med 2003;163:1009–21. doi:10.1001/archinte.163.9.1009.

[9] Comstock GW, Baum C, Snider DE. Isoniazid prophylaxis among Alaskan Eskimos: a final report of the bethel isoniazid studies. Am Rev Respir Dis 1979;119:827–30.

[10] Barry CE, Boshoff HI, Dartois V, Dick T, Ehrt S, Flynn J, Schnappinger D, Wilkinson RJ, Young D. The spectrum of latent tuberculosis: rethinking the biology and intervention strategies. Nat Rev Microbiol 2009;7:845–55. doi:10.1038/nrmicro2236.

[11] Strimbu K, Tavel JA. What are biomarkers? Curr Opin HIV AIDS 2010;5:463–6. doi:10.1097/COH.0b013e32833ed177.

[12] Walzl G, Ronacher K, Hanekom W, Scriba TJ, Zumla A. Immunological biomarkers of tuberculosis. Nat Rev Immunol 2011;11:343–54. doi:10.1038/nri2960.

[13] Chegou NN, Detjen AK, Thiart L, Walters E, Mandalakas AM, Hesseling AC, Walzl G. Utility of host markers detected in Quantiferon supernatants for the diagnosis of tuberculosis in children in a high-burden setting. PLoS One 2013;8:e64226. doi:10.1371/journal.pone.0064226.

[14] Essone PN, Chegou NN, Loxton AG, Stanley K, Kriel M, van der Spuy G, Franken KL, Ottenhoff TH, Walzl G. Host cytokine responses induced after overnight stimulation with novel M. tuberculosis infection phase-dependent antigens show promise as diagnostic candidates for TB disease. PLoS One 2014;9:e102584. doi:10.1371/journal.pone.0102584.

[15] Mihret A, Bekele Y, Bobosha K, Kidd M, Aseffa A, Howe R, Walzl G. Plasma cytokines and chemokines differentiate between active disease and non-active tuberculosis infection. J Infect 2013;66:357–65. doi:10.1016/j.jinf.2012.11.005.

[16] Essone PN, Kalsdorf B, Chegou NN, Loxton AG, Kriel M, Preyer R, Ernst M, Walzl G, Lange C. Bifunctional T-cell-derived cytokines for the diagnosis of tuberculosis and treatment monitoring. Respiration 2014;88:251–61. doi:10.1159/000365816.

[17] Kleinsteuber K, Heesch K, Schattling S, Kohns M, Sander-Jülch C, Walzl G, Hesseling A, Mayatepek E, Fleischer B, Marx FM, Jacobsen M. Decreased expression of miR-21, miR-26a, miR-29a, and miR-142-3p in $CD4^+$ T cells and peripheral blood from tuberculosis patients. PLoS One 2013;8:e61609. doi:10.1371/journal.pone.0061609.

[18] Zhang X, Guo J, Fan S, Li Y, Wei L, Yang X, Jiang T, Chen Z, Wang C, Liu J, Ping Z, Xu D, Wang J, Li Z, Qiu Y, Li J-C. Screening and identification of six serum microRNAs as novel potential combination biomarkers for pulmonary tuberculosis diagnosis. PLoS One 2013;8:e81076. doi:10.1371/journal.pone.0081076.

[19] Abd-El-Fattah AA, Sadik NAH, Shaker OG, Aboulftouh ML. Differential microRNAs expression in serum of patients with lung cancer, pulmonary tuberculosis, and pneumonia. Cell Biochem Biophys 2013;67:875–84. doi:10.1007/s12013-013-9575-y.

[20] Baumann R, Kaempfer S, Chegou NN, Oehlmann W, Loxton AG, Kaufmann SHE, van Helden PD, Black GF, Singh M, Walzl G. Serologic diagnosis of tuberculosis by combining Ig classes against selected mycobacterial targets. J Infect 2014. doi:10.1016/j.jinf.2014.05.014.

[21] Tonegawa S. Somatic generation of antibody diversity. Nature 1983;302:575–81. doi:10.1038/302575a0.

[22] Larosa DF, Orange JS. 1. Lymphocytes. J Allergy Clin Immunol 2008;121:S364–9; quiz S412. doi:10.1016/j.jaci.2007.06.016.

[23] Beck JM. The immunocompromised host: HIV infection. Proc Am Thorac Soc 2005;2:423–7. doi:10.1513/pats.200507-077JS.

[24] Young KR, Rankin JA, Naegel GP, Paul ES, Reynolds HY. Bronchoalveolar lavage cells and proteins in patients with the acquired immunodeficiency syndrome. An immunologic analysis. Ann Intern Med 1985;103:522–33.

[25] Jelicic K, Cimbro R, Nawaz F, Huang DW, Zheng X, Yang J, et al. The HIV-1 envelope protein gp120 impairs B cell proliferation by inducing TGF-β1 production and FcRL4 expression. Nat Immunol 2013;advance on. doi:10.1038/ni.2746.

[26] Metlas R, Srdic T, Jenabian M-A, Elluru S, Carta L, Bowker T, Kaveri S, Concas D. Immunoglobulin G-Reactive Antibodies from Sera of Healthy Individuals Enriched in IgG2 - Therapeutic Potential in HIV-1 Infection. Curr HIV Res 2009;7:378–83. doi:10.2174/157016209788680516.

[27] Buckner CM, Moir S, Ho J, Wang W, Posada JG, Kardava L, Funk EK, Nelson AK, Li Y, Chun T-W, Fauci AS. Characterization of plasmablasts in the blood of HIV-infected viremic individuals: evidence for nonspecific immune activation. J Virol 2013;87:5800–11. doi:10.1128/JVI.00094-13.

[28] Goletti D, Weissman D, Jackson RW, Graham NM, Vlahov D, Klein RS, Munsiff SS, Ortona L, Cauda R, Fauci AS. Effect of Mycobacterium tuberculosis on HIV replication. Role of immune activation. J Immunol 1996;157:1271–8.

[29] Hertoghe T, Wajja A, Ntambi L, Okwera A, Aziz MA, Hirsch C, Johnson J, Toossi Z, Mugerwa R, Mugyenyi P, Colebunders R, Ellner J, Vanham G. T cell activation, apoptosis and cytokine dysregulation in the (co)pathogenesis of HIV and pulmonary tuberculosis (TB). Clin Exp Immunol 2000;122:350–7.

[30] Toossi Z, Johnson JL, Kanost RA, Wu M, Luzze H, Peters P, Okwera A, Joloba M, Mugyenyi P, Mugerwa RD, Aung H, Ellner JJ, Hirsch CS. Increased replication of HIV-1 at sites of Mycobacterium tuberculosis infection: potential mechanisms of viral activation. J Acquir Immune Defic Syndr 2001;28:1–8.

[31] Toossi Z, Mayanja-Kizza H, Hirsch CS, Edmonds KL, Spahlinger T, Hom DL, Aung H, Mugyenyi P, Ellner JJ, Whalen CW. Impact of tuberculosis (TB) on HIV-1 activity in dually infected patients. Clin Exp Immunol 2001;123:233–8.

[32] Vanham G, Edmonds K, Qing L, Hom D, Toossi Z, Jones B, Daley CL, Huebner B, Kestens L, Gigase P, Ellner JJ. Generalized immune activation in pulmonary tuberculosis: co-activation with HIV infection. Clin Exp Immunol 1996;103:30–4.

[33] Mihret A, Abebe M, Bekele Y, Aseffa A, Walzl G, Howe R. Impact of HIV co-infection on plasma level of cytokines and chemokines of pulmonary tuberculosis patients. BMC Infect Dis 2014;14:125. doi:10.1186/1471-2334-14-125.

[34] Allman D, Pillai S. Peripheral B cell subsets. Curr Opin Immunol 2008;20:149–57. doi:10.1016/j.coi.2008.03.014.

[35] Pasare C, Medzhitov R. Control of B-cell responses by Toll-like receptors. Nature 2005;438:364–8. doi:10.1038/nature04267.

[36] Gavin AL, Hoebe K, Duong B, Ota T, Martin C, Beutler B, Nemazee D. Adjuvant-enhanced antibody responses in the absence of toll-like receptor signaling. Science 2006;314:1936–8. doi:10.1126/science.1135299.

[37] Agrawal S, Smith S a BC, Tangye SG, Sewell W a. Transitional B cell subsets in human bone marrow. Clin Exp Immunol 2013;174:53–9. doi:10.1111/cei.12149.

[38] Suryani S, Fulcher D a, Santner-Nanan B, Nanan R, Wong M, Shaw PJ, Gibson J, Williams A, Tangye SG. Differential expression of CD21 identifies developmentally and functionally distinct subsets of human transitional B cells. Blood 2010;115:519–29. doi:10.1182/blood-2009-07-234799.

[39] Marie-Cardine A, Divay F, Dutot I, Green A, Perdrix A, Boyer O, Contentin N, Tilly H, Tron F, Vannier J-P, Jacquot S. Transitional B cells in humans: characterization and insight from B lymphocyte reconstitution after hematopoietic stem cell transplantation. Clin Immunol 2008;127:14–25. doi:10.1016/j.clim.2007.11.013.

[40] Palanichamy A, Barnard J, Zheng B, Owen T, Quach T, Wei C, Looney RJ, Sanz I, Anolik JH. Novel human transitional B cell populations revealed by B cell depletion therapy. J Immunol 2009;182:5982–93. doi:10.4049/jimmunol.0801859.

[41] Vossenkämper A, Blair PA, Safinia N, Fraser LD, Das L, Sanders TJ, Stagg AJ, Sanderson JD, Taylor K, Chang F, Choong LM, D'Cruz DP, Macdonald TT, Lombardi G, Spencer J. A role for

gut-associated lymphoid tissue in shaping the human B cell repertoire. J Exp Med 2013;210:1665–74. doi:10.1084/jem.20122465.

[42] Manz RA, Hauser AE, Hiepe F, Radbruch A. Maintenance of serum antibody levels. Annu Rev Immunol 2005;23:367–86. doi:10.1146/annurev.immunol.23.021704.115723.

[43] Helmreich E, Kern M, Eisen HN. The secretion of antibody by isolated lymph node cells. J Biol Chem 1961;236:464–73.

[44] WHITE RG. Antibody production by single cells. Nature 1958;182:1383–4.

[45] Barr TA, Shen P, Brown S, Lampropoulou V, Roch T, Lawrie S, Fan B, O'Connor RA, Anderton SM, Bar-Or A, Fillatreau S, Gray D. B cell depletion therapy ameliorates autoimmune disease through ablation of IL-6-producing B cells. J Exp Med 2012;209:1001–10. doi:10.1084/jem.20111675.

[46] Shen P, Roch T, Lampropoulou V, O'Connor RA, Stervbo U, Hilgenberg E, et al. IL-35-producing B cells are critical regulators of immunity during autoimmune and infectious diseases. Nature 2014;507:366–70. doi:10.1038/nature12979.

[47] Lund FE. Cytokine-producing B lymphocytes-key regulators of immunity. Curr Opin Immunol 2008;20:332–8. doi:10.1016/j.coi.2008.03.003.

[48] Fillatreau S, Sweenie CH, McGeachy MJ, Gray D, Anderton SM. B cells regulate autoimmunity by provision of IL-10. Nat Immunol 2002;3:944–50. doi:10.1038/ni833.

[49] Harris DP, Haynes L, Sayles PC, Duso DK, Eaton SM, Lepak NM, Johnson LL, Swain SL, Lund FE. Reciprocal regulation of polarized cytokine production by effector B and T cells. Nat Immunol 2000;1:475–82. doi:10.1038/82717.

[50] Mizoguchi A, Bhan AK. A case for regulatory B cells. J Immunol 2006;176:705–10.

[51] Menard LC, Minns LA, Darche S, Mielcarz DW, Foureau DM, Roos D, Dzierszinski F, Kasper LH, Buzoni-Gatel D. B cells amplify IFN-gamma production by T cells via a TNF-alpha-mediated mechanism. J Immunol 2007;179:4857–66.

[52] Neyt K, Perros F, GeurtsvanKessel CH, Hammad H, Lambrecht BN. Tertiary lymphoid organs in infection and autoimmunity. Trends Immunol 2012;33:297–305. doi:10.1016/j.it.2012.04.006.

[53] Martin F, Kearney JF. Marginal-zone B cells. Nat Rev Immunol 2002;2:323–35. doi:10.1038/nri799.

[54] Maglione PJ, Xu J, Casadevall A, Chan J. Fc gamma receptors regulate immune activation and susceptibility during Mycobacterium tuberculosis infection. J Immunol 2008;180:3329–38.

[55] Sutherland JS, Loxton AG, Haks MC, Kassa D, Ambrose L, Lee JS, et al. Differential gene expression of activating Fcγ receptor classifies active tuberculosis regardless of human immunodeficiency virus status or ethnicity. Clin Microbiol Infect 2014;20:O230–8. doi:10.1111/1469-0691.12383.

[56] Rolink AG, Melchers F. BAFFled B cells survive and thrive: roles of BAFF in B-cell development. Curr Opin Immunol 2002;14:266–75. doi:10.1016/S0952-7915(02)00332-1.

[57] MacLennan I, Vinuesa C. Dendritic cells, BAFF, and APRIL: innate players in adaptive antibody responses. Immunity 2002;17:235–8.

[58] Mackay F, Schneider P, Rennert P, Browning J. BAFF AND APRIL: a tutorial on B cell survival. Annu Rev Immunol 2003;21:231–64. doi:10.1146/annurev.immunol.21.120601.141152.

[59] Gross JA, Dillon SR, Mudri S, Johnston J, Littau A, Roque R, Rixon M, Schou O, Foley KP, Haugen H, McMillen S, Waggie K, Schreckhise RW, Shoemaker K, Vu T, Moore M, Grossman A, Clegg CH. TACI-Ig neutralizes molecules critical for B cell development and autoimmune disease. impaired B cell maturation in mice lacking BLyS. Immunity 2001;15:289–302.

[60] Schiemann B, Gommerman JL, Vora K, Cachero TG, Shulga-Morskaya S, Dobles M, Frew E, Scott ML. An essential role for BAFF in the normal development of B cells through a BCMA-independent pathway. Science 2001;293:2111–4. doi:10.1126/science.1061964.

[61] Thompson JS, Bixler SA, Qian F, Vora K, Scott ML, Cachero TG, Hession C, Schneider P, Sizing ID, Mullen C, Strauch K, Zafari M, Benjamin CD, Tschopp J, Browning JL, Ambrose C. BAFF-R, a newly identified TNF receptor that specifically interacts with BAFF. Science 2001;293:2108–11. doi:10.1126/science.1061965.

[62] Liu K, Zhang Y, Hu S, Yu Y, Yang Q, Jin D, Chen X, Jin Q, Liu H. Increased levels of BAFF and APRIL related to human active pulmonary tuberculosis. PLoS One 2012;7:e38429. doi:10.1371/journal.pone.0038429.

[63] Billingham RE, Brent L, Medawar PB. Actively acquired tolerance of foreign cells. Nature 1953;172:603–6.

[64] Koenig S, Hoffmann MK. Bacterial lipopolysaccharide activates suppressor B lymphocytes. Proc Natl Acad Sci U S A 1979;76:4608–12.

[65] Feldmann M, Nossal GJ. Tolerance, enhancement and the regulation of interactions between T cells, B cells and macrophages. Transplant Rev 1972;13:3–34.

[66] Lampropoulou V, Hoehlig K, Roch T, Neves P, Calderón Gómez E, Sweenie CH, Hao Y, Freitas AA, Steinhoff U, Anderton SM, Fillatreau S. TLR-activated B cells suppress T cell-mediated autoimmunity. J Immunol 2008;180:4763–73.

[67] Hoehlig K, Lampropoulou V, Roch T, Neves P, Calderon-Gomez E, Anderton SM, Steinhoff U, Fillatreau S. Immune regulation by B cells and antibodies a view towards the clinic. Adv Immunol 2008;98:1–38. doi:10.1016/S0065-2776(08)00401-X.

[68] Neves P, Lampropoulou V, Calderon-Gomez E, Roch T, Stervbo U, Shen P, Kühl AA, Loddenkemper C, Haury M, Nedospasov SA, Kaufmann SHE, Steinhoff U, Calado DP, Fillatreau S. Signaling via the MyD88 adaptor protein in B cells suppresses protective immunity during Salmonella typhimurium infection. Immunity 2010;33:777–90. doi:10.1016/j.immuni.2010.10.016.

[69] Mizoguchi A, Mizoguchi E, Takedatsu H, Blumberg RS, Bhan AK. Chronic intestinal inflammatory condition generates IL-10-producing regulatory B cell subset characterized by CD1d upregulation. Immunity 2002;16:219–30.

[70] Mauri C, Gray D, Mushtaq N, Londei M. Prevention of arthritis by interleukin 10-producing B cells. J Exp Med 2003;197:489–501.

[71] Fillatreau S, Gray D, Anderton SM. Not always the bad guys: B cells as regulators of autoimmune pathology. Nat Rev Immunol 2008;8:391–7. doi:10.1038/nri2315.

[72] Anderton SM, Fillatreau S. Activated B cells in autoimmune diseases: the case for a regulatory role. Nat Clin Pract Rheumatol 2008;4:657–66. doi:10.1038/ncprheum0950.

[73] Lampropoulou V, Calderon-Gomez E, Roch T, Neves P, Shen P, Stervbo U, Boudinot P, Anderton SM, Fillatreau S. Suppressive functions of activated B cells in autoimmune diseases reveal the dual roles of Toll-like receptors in immunity. Immunol Rev 2010;233:146–61. doi:10.1111/j.0105-2896.2009.00855.x.

[74] Macpherson AJ, Uhr T. Induction of protective IgA by intestinal dendritic cells carrying commensal bacteria. Science 2004;303:1662–5. doi:10.1126/science.1091334.

[75] Fagarasan S, Kawamoto S, Kanagawa O, Suzuki K. Adaptive immune regulation in the gut: T cell-dependent and T cell-independent IgA synthesis. Annu Rev Immunol 2010;28:243–73. doi:10.1146/annurev-immunol-030409-101314.

[76] Tezuka H, Abe Y, Iwata M, Takeuchi H, Ishikawa H, Matsushita M, Shiohara T, Akira S, Ohteki T. Regulation of IgA production by naturally occurring TNF/iNOS-producing dendritic cells. Nature 2007;448:929–33. doi:10.1038/nature06033.

[77] Fritz JH, Rojas OL, Simard N, McCarthy DD, Hapfelmeier S, Rubino S, Robertson SJ, Larijani M, Gosselin J, Ivanov II, Martin A, Casellas R, Philpott DJ, Girardin SE, McCoy KD, Macpherson AJ, Paige CJ, Gommerman JL. Acquisition of a multifunctional IgA+ plasma cell phenotype in the gut. Nature 2012;481:199–203. doi:10.1038/nature10698.

[78] Dang VD, Hilgenberg E, Ries S, Shen P, Fillatreau S. From the regulatory functions of B cells to the identification of cytokine-producing plasma cell subsets. Curr Opin Immunol 2014;28C:77–83. doi:10.1016/j.coi.2014.02.009.

[79] Maaser C, Housley MP, Iimura M, Smith JR, Vallance BA, Finlay BB, Schreiber JR, Varki NM, Kagnoff MF, Eckmann L. Clearance of Citrobacter rodentium requires B cells but not secretory immunoglobulin A (IgA) or IgM antibodies. Infect Immun 2004;72:3315–24. doi:10.1128/IAI.72.6.3315-3324.2004.

[80] Bermejo DA, Jackson SW, Gorosito-Serran M, Acosta-Rodriguez E V, Amezcua-Vesely MC, Sather BD, Singh AK, Khim S, Mucci J, Liggitt D, Campetella O, Oukka M, Gruppi A, Rawlings DJ. Trypanosoma cruzi trans-sialidase initiates a program independent of the transcription factors RORγt and Ahr that leads to IL-17 production by activated B cells. Nat Immunol 2013;14:514–22. doi:10.1038/ni.2569.

[81] Martin GS, Mannino DM, Eaton S, Moss M. The epidemiology of sepsis in the United States from 1979 through 2000. N Engl J Med 2003;348:1546–54. doi:10.1056/NEJMoa022139.

[82] Beutler B, Milsark IW, Cerami AC. Passive immunization against cachectin/tumor necrosis factor protects mice from lethal effect of endotoxin. Science, 1985, 229(4716):869-871. Classical article. J Immunol 2008;181:7–9.

[83] Hotchkiss RS, Coopersmith CM, McDunn JE, Ferguson TA. The sepsis seesaw: tilting toward immunosuppression. Nat Med 2009;15:496–7. doi:10.1038/nm0509-496.

[84] Kelly-Scumpia KM, Scumpia PO, Weinstein JS, Delano MJ, Cuenca AG, Nacionales DC, Wynn JL, Lee PY, Kumagai Y, Efron PA, Akira S, Wasserfall C, Atkinson MA, Moldawer LL. B cells enhance early innate immune responses during bacterial sepsis. J Exp Med 2011;208:1673–82. doi:10.1084/jem.20101715.

[85] Rauch PJ, Chudnovskiy A, Robbins CS, Weber GF, Etzrodt M, Hilgendorf I, Tiglao E, Figueiredo J-L, Iwamoto Y, Theurl I, Gorbatov R, Waring MT, Chicoine AT, Mouded M, Pittet MJ, Nahrendorf M, Weissleder R, Swirski FK. Innate response activator B cells protect against microbial sepsis. Science 2012;335:597–601. doi:10.1126/science.1215173.

[86] Hamilton JA. Colony-stimulating factors in inflammation and autoimmunity. Nat Rev Immunol 2008;8:533–44. doi:10.1038/nri2356.

[87] Sonderegger I, Iezzi G, Maier R, Schmitz N, Kurrer M, Kopf M. GM-CSF mediates autoimmunity by enhancing IL-6-dependent Th17 cell development and survival. J Exp Med 2008;205:2281–94. doi:10.1084/jem.20071119.

[88] Mueller H, Detjen AK, Schuck SD, Gutschmidt A, Wahn U, Magdorf K, Kaufmann SHE, Jacobsen M. Mycobacterium tuberculosis-specific CD4+, IFNgamma+, and TNFalpha+ multifunctional memory T cells coexpress GM-CSF. Cytokine 2008;43:143–8. doi:10.1016/j.cyto.2008.05.002.

[89] Weber GF, Chousterman BG, Hilgendorf I, Robbins CS, Theurl I, Gerhardt LMS, Iwamoto Y, Quach TD, Ali M, Chen JW, Rothstein TL, Nahrendorf M, Weissleder R, Swirski FK. Pleural innate response activator B cells protect against pneumonia via a GM-CSF-IgM axis. J Exp Med 2014;211:1243–56. doi:10.1084/jem.20131471.

[90] de Wit J, Jorritsma T, Makuch M, Remmerswaal EBM, Klaasse Bos H, Souwer Y, Neefjes J, Ten Berge IJM, van Ham SM. Human B cells promote T-cell plasticity to optimize antibody response by inducing coexpression of TH1/TFH signatures. J Allergy Clin Immunol 2014. doi:10.1016/j.jaci.2014.08.012.

[91] Jones DD, Jones M, Deiulio GA, Racine R, Macnamara KC, Winslow GM. B Cell Activating Factor Inhibition Impairs Bacterial Immunity by Reducing T Cell-Independent IgM Secretion. Infect Immun 2013;81:4490–7. doi:10.1128/IAI.00998-13.

[92] Ma N, He Y, Xiao H, Han G, Chen G, Wang Y, Wang K, Hou C, Yang X, Marrero B, Wang Y, Shen B, Li Y, Wang R. BAFF maintains T-cell survival by inducing OPN expression in B cells. Mol Immunol 2014;57:129–37. doi:10.1016/j.molimm.2013.08.014.

[93] Maglione PJ, Xu J, Chan J. B cells moderate inflammatory progression and enhance bacterial containment upon pulmonary challenge with Mycobacterium tuberculosis. J Immunol 2007;178:7222–34.

[94] Tsitsiou E, Lindsay MA. microRNAs and the immune response. Curr Opin Pharmacol 2009;9:514–20. doi:10.1016/j.coph.2009.05.003.

[95] Winter J, Jung S, Keller S, Gregory RI, Diederichs S. Many roads to maturity: microRNA biogenesis pathways and their regulation. Nat Cell Biol 2009;11:228–34. doi:10.1038/ncb0309-228.

[96] Eulalio A, Huntzinger E, Izaurralde E. Getting to the root of miRNA-mediated gene silencing. Cell 2008;132:9–14. doi:10.1016/j.cell.2007.12.024.

[97] Filipowicz W, Bhattacharyya SN, Sonenberg N. Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? Nat Rev Genet 2008;9:102–14. doi:10.1038/nrg2290.

[98] Jardin F, Figeac M. MicroRNAs in lymphoma, from diagnosis to targeted therapy. Curr Opin Oncol 2013;25:480–6. doi:10.1097/CCO.0b013e328363def2.

[99] Hwang H-W, Mendell JT. MicroRNAs in cell proliferation, cell death, and tumorigenesis. Br J Cancer 2006;94:776–80. doi:10.1038/sj.bjc.6603023.

[100] Fernando TR, Rodriguez-Malave NI, Rao DS. MicroRNAs in B cell development and malignancy. J Hematol Oncol 2012;5:7. doi:10.1186/1756-8722-5-7.

[101] Zhu M, Xu Z, Wang K, Wang N, Zhu M, Wang S. MicroRNA and gene networks in human Hodgkin's lymphoma. Mol Med Rep 2013;8:1747–54. doi:10.3892/mmr.2013.1741.

[102] Goodall EF, Heath PR, Bandmann O, Kirby J, Shaw PJ. Neuronal dark matter: the emerging role of microRNAs in neurodegeneration. Front Cell Neurosci 2013;7:178. doi:10.3389/fncel.2013.00178.

[103] Griffiths-Jones S. miRBase: the microRNA sequence database. Methods Mol Biol 2006;342:129–38. doi:10.1385/1-59745-123-1:129.

[104] Griffiths-Jones S, Grocock RJ, van Dongen S, Bateman A, Enright AJ. miRBase: microRNA sequences, targets and gene nomenclature. Nucleic Acids Res 2006;34:D140–4. doi:10.1093/nar/gkj112.

[105] Jensen K, Brusletto BS, Aass HCD, Olstad OK, Kierulf P, Gautvik KM. Transcriptional profiling of mRNAs and microRNAs in human bone marrow precursor B cells identifies subset- and age-specific variations. PLoS One 2013;8:e70721. doi:10.1371/journal.pone.0070721.

[106] Miyazaki Y, Li R, Rezk A, Misirliyan H, Moore C, Farooqi N, Solis M, Goiry LG, de Faria Junior O, Dang VD, Colman D, Dhaunchak AS, Antel J, Gommerman J, Prat A, Fillatreau S, Bar-Or

A. A Novel MicroRNA-132-Surtuin-1 Axis Underlies Aberrant B-cell Cytokine Regulation in Patients with Relapsing-Remitting Multiple Sclerosis. PLoS One 2014;9:e105421. doi:10.1371/journal.pone.0105421.

Chapter 2:

Unique peripheral B cell populations during Tuberculosis infection and disease

The work presented in this chapter has been formatted in the style of Immunobiology journal to which it was submitted and is currently under review.

Unique peripheral B cell populations during Tuberculosis infection and disease

Willem J du Plessis[1], Alana Keyser[2], Gerhard Walzl[1] and André G Loxton[1]

1. SA MRC Centre for TB Research, DST/NRF Centre of Excellence for Biomedical Tuberculosis Research, Division of Molecular Biology and Human Genetics, Faculty of Medicine and Health Sciences, Stellenbosch University, Cape Town, South Africa

PO Box 241 Cape Town, 8000 South Africa

2. Clinical Laboratory Sciences, Faculty of Health Sciences, University of Cape Town, Cape Town, South Africa

Corresponding Author:

Dr AG Loxton

Email: GL2@sun.ac.za

Tel: (+27)-21 9389399

Fax: (+27) 21 9389863

Abstract

Mycobacterium tuberculosis (*Mtb*) remains an unresolved threat resulting in great annual loss of life. The role of B cells during the protective immunity to *Mtb* is still unclear. B cells have been described as effector molecules in addition to their role as antibody producing cells during disease. Here we aim to identify and characterize the frequency of peripheral B-cell subpopulations during active Tuberculosis and over treatment response. Analysis were done for both class switched (CS) and non-class switched (NCS) phenotypes. We recruited participants with active untreated pulmonary Tuberculosis, other lung diseases and healthy community controls. All groups were followed up for one week from recruitment and the TB cases till the end of treatment (month 6). Peripheral blood samples were collected, stained with monoclonal antibodies to $CD19^{+}$ cells, Immunoglobulin (Ig) M, plasma cells ($CD\ 138^{+}$), marker of memory ($CD27^{+}$), immune activation ($CD23^{+}$) and acquired on a flow cytometer. Marginal zone B cells ($CD19^{+}IgM^{+}CD23^{-}CD27^{+}$) and memory phenotypes are able to distinguish between TB diagnosis and end of treatment. The frequency of mature B cells from TB cases are lower than that of other-lung diseases at diagnosis. A subpopulation of activated memory B cells ($CD19^{+}IgM^{+}CD23^{+}CD27^{+}$) cells are present at the end of TB treatment. This study identified distinctive B cell subpopulations present during active TB disease and other lung disease conditions. These cell populations warrant further examination in larger studies as it may be informative as cell markers or as effectors/regulators in TB disease or TB treatment response.

Keywords

Keywords: B cells, Marginal zone, Plasma cells, Immuno-phenotyping, biomarker, immune activation

Introduction

Tuberculosis (TB), remains an unresolved threat that is responsible for great mortality and morbidity in humans. Its causative agent, *Mycobacterium tuberculosis* (*Mtb*), was ultimately responsible for 9 million newly reported cases and 1.5 million deaths during 2013 [1]. Although great progress has been made on T cell based tuberculosis research, it is imperative that new avenues are explored and that previously underappreciated cell types are re-evaluated for their roles during the tuberculosis infection with the expectation of bringing an end to the epidemic. It is commonly accepted that B cell and antibody-mediated responses confers protection against extracellular pathogens and that the regulation and control of intracellular organisms are through cellular immune mechanisms.

There is increasing evidence that demonstrates B cells functioning as mediators (in both effector and regulatory roles) of immunity outside of their classically designated profession as the facilitators of humoral immunity. B cell activation by Toll-like receptor (TLR) antigens or whole organisms (like BCG or *Mtb*) can lead to a range of outcomes to the host, either by producing antibody, secreting cytokines (including interleukin (IL)-6, IL-10, and interferon(IFN)-gamma) or presenting antigen to naïve T cells [2–5]. B cell responses are beneficial to the host during infections and damaging during autoimmune disease. Conversely, B cells have the capacity to limit the hosts defence (inflammatory response) against pathogens and shield against autoimmune pathologies. This demonstrates that B cells can have distinct roles as drivers and regulators of immunity depending on the functional properties they gain following receptor activation and differentiation.

Although ongoing studies and literature supports the functional role of B cells during TB, the respective change in the frequency of the circulating B cell repertoire during active TB disease remains a topic for discussion as some studies report either a significant decrease [6] or increase [7] of peripheral blood B cell populations in actively infected patients.

Immuno-phenotyping has proven to be a very useful tool in the identification, monitoring and management of various clinical diseases [8–10]. Although recent publications have sought to develop in depth multicolour flow cytometric panels for the accurate delineation of various lymphocyte populations and subpopulations (including B cells) during immunodeficiency's [11,12], very few studies exist that specifically assesse immune-phenotypic change during active *Mycobacterium tuberculosis* infection [6,13]. Little is known about the immune-phenotypic change of the B cell lineage during active TB disease as current literature largely focusses on the general B cell presence (primarily looking at $CD19^+$ B cells only) [6,13], rather than an in-depth analysis of various populations and subpopulations. This results in a lack of knowledge pertaining to changes in B cell populations implicated in effector roles such as circulating memory B cells or plasma populations. It also does not elucidate the current activation state of B cells nor the expression of surface molecules, thus highlighting the need for further investigation regarding this matter.

In this report, a total of 96 participant samples spanning three groups (tuberculosis – active disease; 52 samples, other-lung disease; 24 samples, and healthy community controls; 20 samples) and various time points relating

to treatment were used to assess the B cell repertoire in detail with the hope of identifying unique phenotypic differences between the groups that could suffice as biomarkers of disease. The primary contribution of this data would be to map the phenotypic distribution of B cells between these groups with a vast range, as it would include phenotypes for both IgM^+ and IgM^- B cells. The actual isotype linked to the IgM^- phenotypes is contestable, but it is likely to be represented by IgD^+ populations.

Materials and Methods

Ethics Statement

All participants gave written informed consent for partaking in the study after being briefed about the study's aims and goals. The participants were also required to have their HIV status tested or declared to field workers. Samples were collected from two TB treatment studies. This study was carried out according to the Declaration of Helsinki and ethical approval was obtained for two studies utilised for sample collection from the Health Research Ethics Committee of Stellenbosch University with ethics reference #: N10/01/013 and N13/05/064.

Patients

This study was done in the Western Cape Province of South Africa where a 2003 report showed that the TB detection rate was 678/100'000 population[14]. Previous publications have defined the characteristics associated with the economically depressed and disadvantaged metropolitan population of mixed ancestry primarily found in the Western Cape Province, presenting high incidence rates of *Mtb* and transmission[15,16]. The study participants included attendees of the Infectious Diseases Clinics at Tygerberg Hospital, community members of surrounding areas including Ravensmead, Uitsig, Adriaanse, Elsiesriver and visitor of local health care clinics. A total of 96 HIV negative participants, spanning three groups, were recruited for this study. Fifty-two had active tuberculosis disease (TB disease status was confirmed by two separate positive sputum smear tests and a PCR for DNA of bacteria of the *Mtb* complex, by utilising the GeneXpert platform), 20 were healthy community controls and the third group consisted of 24 other-lung disease (OLD) patients. These OLD patients were all TB and HIV negative and presented with at least one of the following: a) febrile illness with chest symptoms, b) radiographic evidence of viral or bacterial pneumonia, c) bronchiectasis with acute exacerbation, or d) acute exacerbation of asthma or COPD (chronic obstructive pulmonary disease). Table 1 summarizes the demographic data of study participants.

B cell Phenotyping

Peripheral blood samples from the two control groups and patients were collected on various scheduled visits which included diagnosis, day 7 on treatment and at week 24 (end of TB treatment). White blood cells were obtained by subjecting each sample to a red blood cell lysing step using BD FACSlyse solution (BD Bioscience Pharmingen – San Jose, CA, USA). Leukocytes were stained following a standard procedure with anti-human CD3 (APC/Cy7, clone HIT3a), anti-human CD4 (PerCP/Cy5.5, clone OKT4), anti-human CD8a (Brilliant Violet 510, clone RPA-T8), anti-human CD19 (PE/Cy7, clone HIB19), anti-human CD23 (FITC, clone EBVCS-5), anti-human CD27 (PE, clone M-T271), anti-human CD138 (APC, clone DL-101) and anti-human IgM (Brilliant Violet, clone MHM-88). All antibodies were purchased from BioLegend (San Diego, California, United States of America). A total of 100 000 lymphocytes/sample were acquired on a FACS Canto II (BD Biosciences). All post acquisition analysis was done with FlowJo Software v10 (Tree Star Inc.).

The assessed phenotypes (Figure 1) were defined as follows: (1) Mature B cells ($CD19^{+}IgM^{+}$), (2) Activated B cells ($CD19^{+}IgM^{+}CD23^{+}$), (3) Naïve B cells ($CD19^{+}IgM^{+}CD23^{+}CD27^{+}$), (4) Marginal Zone (MZ) B cells ($CD19^{+}IgM^{+}CD23^{-}CD27^{+}$), (5) Memory B cells ($CD19^{+}IgM^{+}CD27^{+}$), (6) $Memory^{high}$ B cells ($CD19^{+}IgM^{+}CD27^{++}$) (7) Total plasma cells ($CD19^{+}IgM^{+}CD138^{+}$), (8) Memory-Plasma cells ($CD19^{+}IgM^{+}CD27^{+}CD138^{+}$) and (9) $Memory^{high}$-plasma B cells ($CD19^{+}IgM^{+}CD138^{+}CD27^{++}$). All IgM^{+} phenotypes are referred to as class switched (CS) and IgM^{-} phenotypes as non-class switched (NCS) in text.

Statistical Analysis

Differences in the frequency of B cell subsets between the groups were analysed using the non-parametric analysis with a Mann-Whitney correction and performed by Dr Justin Harvey (Stellenbosch University). All analysis was performed with the Statistica 12 software (Statsoft, Ohio, USA).

Results/Discussion

Marginal zone B cells and memory phenotypes distinguish between TB diagnosis and end of treatment.

We firstly aimed to identify phenotypes that were significantly different at diagnosis and the end of treatment (week 24). In Figure 2 it is seen that the CD27high memory B cells (CD19+IgM+CD27++), p = 0.02880, and memory-plasma B cells (CD19+IgM+CD138+CD27+), p = 0.00389, populations were significantly higher at diagnosis when compared to levels at the end of treatment. Sebina et al. [17] observed that UK donors who previously lived in, or visited areas denoted as highly TB-endemic had higher frequencies of memory B cells in their peripheral blood as compared to their counterparts. This study also reported that BCG elicited the production of long-lived mycobacteria-specific memory B cells [17], which supports the notion of high frequencies of memory B cells at diagnosis as BCG vaccination is a common practice at birth in South Africa. It has been shown that the maintenance of memory B cells is dependent on the presence of antigen, and that these B cells are lost within 10 – 12 weeks following its absence [18]. Although this is contrary to other publications stating the longevity of B cells [17,19–21], it corresponds to the observed result of lower memory B cell frequencies at the end of treatment. It is reasoned that the decrease of memory B cells towards the end of treatment does not relate to a complete loss of memory B cells, but rather represents an overall decrease in line with the reduction of bacterial burden. Figure 3 shows that memory based B cell phenotypes were significant in the NCS cohort as well with memory B cells (CD19+IgM-CD27+); p = 0.01398, memory-plasma B cells (CD19+IgM-CD138+CD27+); p = 0.00968 and memory^{high}-plasma (CD19+IgM-CD138+CD27++); p = 0.03616. This continual significance in both CS and NCS phenotypes strengthens their importance as distinguishing factors. Marginal zone B cells (CD19+CD27+CD23-) were also significant in both CS (p = 0.04680) and NCS (p = 0.02138) analyses and between groups over time (Figure 4). Together these results warrant further research into these phenotypes as potential biomarkers for treatment response.

Marginal zone- and Mature B cells can distinguish TB from other-lung diseases at diagnosis.

In the attempt to identify phenotypes that were unique to tuberculosis when compared to other-lung based diseases, two showed to be significant. The first was CS marginal zone (MZ) B cells (CD19+IgM+CD27+CD23-) with p = 0.02092 and secondly NCS mature B cells were significant with p = 0.00026 (Figure 4). With both of these phenotypes significantly lower in peripheral circulation during active TB disease (especially the NCS mature B cells), it raises the question whether TB actively suppresses the B cell repertoire during disease. The NCS mature B cell repertoire does not recover to baseline levels during the first week of treatment (in line with chemotherapeutic treatment alleviating bacterial burden). These findings support the notion that there is a possible underlying mechanism exploited by *Mtb* that could be crucial for the management of the infection as both MZ and mature B cells are implicated in effector functions of the adaptive

immune system, as seen with the overexpression of programmed death 1 (PD-1) on lymphocyte frequencies during active TB infection [22].

Class switched and non-class switched mature B cells distinguish between tuberculosis, other-lung based diseases and healthy controls.

Class switched mature B cells from participants with active TB are present in significantly different levels when compared to other-lung diseases ($p = 0.043711$) and the healthy control group ($p = 0.024488$) (Figure 5). Mature B cells were not only able to distinguish between these groups in the class switched category, but also in the non-class switched category where the difference between TB and OLD was highly significant ($p = 0.000016$) and between TB and the healthy control group where $p = 0.025939$ (not shown)). An interesting observation is that the class switched mature B cells from TB are present at higher frequencies when compared to the OLD group (Figure 5), but that the inverse is observed with non-class switched mature B cells where they are present in significantly lower levels when compared to the OLD group (not shown). This would suggest that a strong preference is displayed towards class switched mature B cells during active tuberculosis. The differences present in the CS and NCS mature B cells are consistent when compared during the first week of treatment (not shown), where the NCS mature B cells are present in higher frequencies as when compared to the class switched mature B cells. Further in this study, CS activated B cells in TB could distinguish from OLD ($p = 0.008391$), but not from healthy controls ($p = 0.122877$) (Figure 6). This result identifies another combination of markers that could be used to distinguish TB from other lung based diseases and should be further investigated as a measure in the early diagnosis of TB.

.

Conclusion

This pilot study identified unique variations in the B cell repertoire during tuberculosis disease when compared to healthy controls, other-lung diseases and over the course of TB treatment. The first observation of memory-based phenotypes being the major distinguishers between diagnosis and end of treatment in both class switched and non-class switched phenotypes holds promise as markers for treatment response. The second important finding of this study is that marginal zone B cells could not only distinguish between TB diagnosis and the end of treatment, but also has significantly different frequencies when compared to other-lung based diseases making it a candidate as biomarker for not only treatment response, but distinguishing active TB disease from other-lung based diseases. The observation that CS and NCS mature B cells could best distinguish between TB and the two control groups (healthy controls and other lung diseases) at diagnosis, but that their respective peripheral frequencies are present at an inverse level. Taken together, these results show that mainly B cell phenotypes implicated in activation and subsequent effector functions are influenced by TB and warrants further research to confirm their potential as biomarkers for TB disease and treatment response. These results are further strengthened if the assumption holds that only 10% of the ongoing immunologic response is represented in the peripheral blood when compared to the site of disease, namely the lungs. Further studies are thus needed to replicate this experiment on broncho-alveolar lavage (BAL) fluid from active TB diseased participants and other lung diseases to ascertain if the phenotype remains the same. Although the results from the BAL fluid experiment might give a better representation of the on-site B cell frequencies, the challenges associated with obtaining the bio fluid and characterisation of the (mostly activated) cells could hamper the process of finding biomarkers of disease or TB treatment response.

Acknowledgements

Special thank you to all the study participants and field staff of the Immunology Research Group (SUNIRG). The financial assistance of the National Research Foundation (NRF) towards the research project of WJ du Plessis is hereby acknowledged. Opinions expressed and conclusions arrived at, are those of the authors and are not to be attributed to the NRF. The authors declare no conflict of interest.

References

[1] WHO. Global Tuberculosis Report 2014. World Heal Organ 2014. http://www.who.int/tb/publications/gloabl_report/en/.

[2] Harris DP, Haynes L, Sayles PC, Duso DK, Eaton SM, Lepak NM, et al. Reciprocal regulation of polarized cytokine production by effector B and T cells. Nat Immunol 2000;1:475–82. doi:10.1038/82717.

[3] Lampropoulou V, Hoehlig K, Roch T, Neves P, Calderón Gómez E, Sweenie CH, et al. TLR-activated B cells suppress T cell-mediated autoimmunity. J Immunol 2008;180:4763–73.

[4] Hoehlig K, Lampropoulou V, Roch T, Neves P, Calderon-Gomez E, Anderton SM, et al. Immune regulation by B cells and antibodies a view towards the clinic. Adv Immunol 2008;98:1–38. doi:10.1016/S0065-2776(08)00401-X.

[5] Neves P, Lampropoulou V, Calderon-Gomez E, Roch T, Stervbo U, Shen P, et al. Signaling via the MyD88 adaptor protein in B cells suppresses protective immunity during Salmonella typhimurium infection. Immunity 2010;33:777–90. doi:10.1016/j.immuni.2010.10.016.

[6] Hernandez J, Velazquez C, Valenzuela O, Robles-Zepeda R, Ruiz-Bustos E, Navarro M, et al. Low number of peripheral blood B lymphocytes in patients with pulmonary tuberculosis. Immunol Invest 2010;39:197–205. doi:10.3109/08820130903586346.

[7] Wu YE, Zhang SW, Peng WG, Li KS, Li K, Jiang JK, et al. Changes in lymphocyte subsets in the peripheral blood of patients with active pulmonary tuberculosis. J Int Med Res 2009;37:1742–9.

[8] Hrušák O, Basso G, Ratei R, Gaipa G, Luria D, Mejstříková E, et al. Flow diagnostics essential code: a simple and brief format for the summary of leukemia phenotyping. Cytometry B Clin Cytom 2014;86:288–91.

[9] Orfao A, Schmitz G, Brando B, Ruiz-Arguelles A, Basso G, Braylan R, et al. Clinically Useful Information Provided by the Flow Cytometric Immunophenotyping of Hematological Malignancies: Current Status and Future Directions. Clin Chem 1999;45:1708–17.

[10] Subasic D, Karamehic J, Delic-Sarac M, Kasumovic M, Mekic M, Eminovic I, et al. Monitoring of disease biomarkers activity and immunophenotyping as important factors in SLE clinical management. Med Arch (Sarajevo, Bosnia Herzegovina) 2012;66:336–9.

[11] Boldt A, Borte S, Fricke S, Kentouche K, Emmrich F, Borte M, et al. Eight-color immunophenotyping of T-, B-, and NK-cell subpopulations for characterization of chronic immunodeficiencies. Cytom Part B - Clin Cytom 2014;206:191–206. doi:10.1002/cyto.b.21162.

[12] Abreu MT, Carvalheiro H, Rodrigues-Sousa T, Domingos A, Segorbe-Luis A, Rodrigues-Santos P, et al. Alterations in the peripheral blood B cell subpopulations of multidrug-resistant tuberculosis patients. Clin Exp Med 2013. doi:10.1007/s10238-013-0258-1.

[13] Barcelos W, Martins-Filho OA, Guimarães TMPD, Oliveira MHP, Spíndola-de-Miranda S, Carvalho BN, et al. Peripheral blood mononuclear cells immunophenotyping in pulmonary tuberculosis patients before and after treatment. Microbiol Immunol 2006;50:597–605.

[14] Partnership A, Metropole C. TB Control in Cape Town - Progress Report 1997-2003 2003.

[15] Classen CN, Warren R, Richardson M, Hauman JH, Gie RP, Ellis JHP, et al. Impact of social interactions in the community on the transmission of tuberculosis in a high incidence area. Thorax 1999;54:136–40. doi:10.1136/thx.54.2.136.

[16] Verver S, Warren RM, Munch Z, Vynnycky E, van Helden PD, Richardson M, et al. Transmission of tuberculosis in a high incidence urban community in South Africa. Int J Epidemiol 2004;33:351–7. doi:10.1093/ije/dyh021.

[17] Sebina I, Cliff JM, Smith SG, Nogaro S, Webb EL, Riley EM, et al. Long-lived memory B-cell responses following BCG vaccination. PLoS One 2012;7:e51381. doi:10.1371/journal.pone.0051381.

[18] Gray D, Skarvall H. B-cell memory is short-lived in the absence of antigen. Nature 1988;336:70–3. doi:10.1084/jem.174.5.969.

[19] Wipasa J, Suphavilai C, Okell LC, Cook J, Corran PH, Thaikla K, et al. Long-lived antibody and B Cell memory responses to the human malaria parasites, Plasmodium falciparum and Plasmodium vivax. PLoS Pathog 2010;6:e1000770. doi:10.1371/journal.ppat.1000770.

[20] Ward SM, Phalora P, Bradshaw D, Leyendeckers H, Klenerman P. Direct ex vivo evaluation of long-lived protective antiviral memory B cell responses against hepatitis B virus. J Infect Dis 2008;198:813–7. doi:10.1086/591094.

[21] Schittek B, Rajewsky K. Maintenance of B-cell memory by long-lived cells generated from proliferating precursors. Nature 1990;346:749–51. doi:10.1038/346749a0.

[22] Singh A, Mohan A, Dey AB, Mitra DK. Inhibiting the programmed death 1 pathway rescues Mycobacterium tuberculosis-specific interferon γ-producing T cells from apoptosis in patients with pulmonary tuberculosis. J Infect Dis 2013;208:603–15. doi:10.1093/infdis/jit206.

Figures and Tables

Table 1. Clinical and demographic data of the study participants

Group	Age Range	Gender (Male/Female)	BMI	HIV (+)	Other-lung Disease Diagnosis[a]	TB Type
Tuberculosis (n = 21)	18 - 50	12M : 9F	17.8 ± 1.7	0	n/a	Pulmonary
HC (n = 14)	19 - 50	12M : 2F	22.9 ± 6.3	0	n/a	n/a
OLD (n = 14)	25 - 64	7M : 7F	27.4 ± 8.2	0	Pneumonia (*n* = 10) Asthma (*n* = 2) COPD (*n* = 1) Pleural Effusion, Reactive (*n* = 1)	n/a

OLD other-lung disease, HC healthy community control, BMI body mass index, COPD chronic obstruction of airways disease. [a] All cases had to include symptoms of fever, coughing and other symptoms that would classify the case as acute and of exacerbated nature. Patients on systemic steroid therapy was excluded from the study.

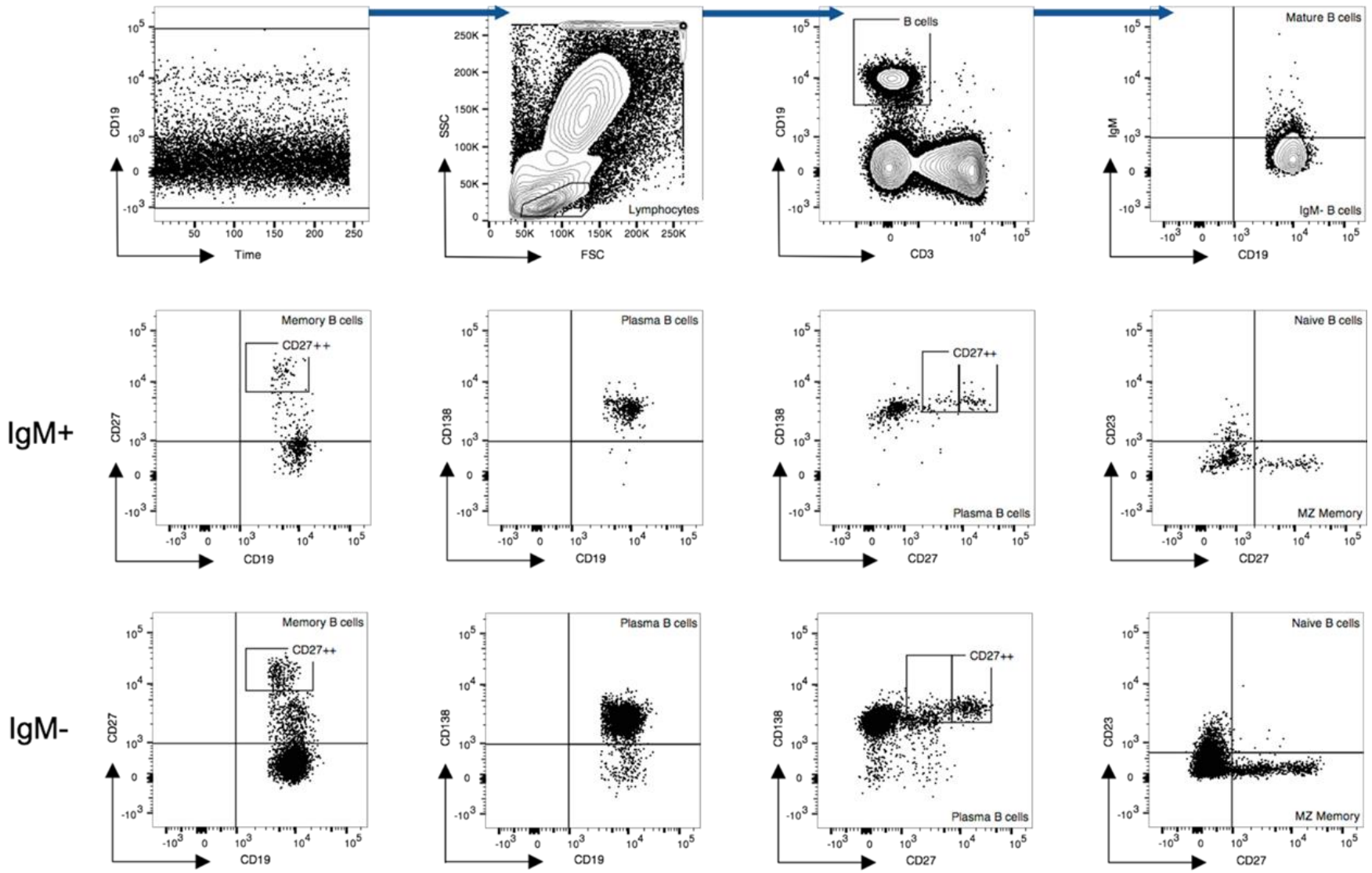


Figure 1. Flow cytometric gating strategy to identify the different peripheral B cell phenotypes.

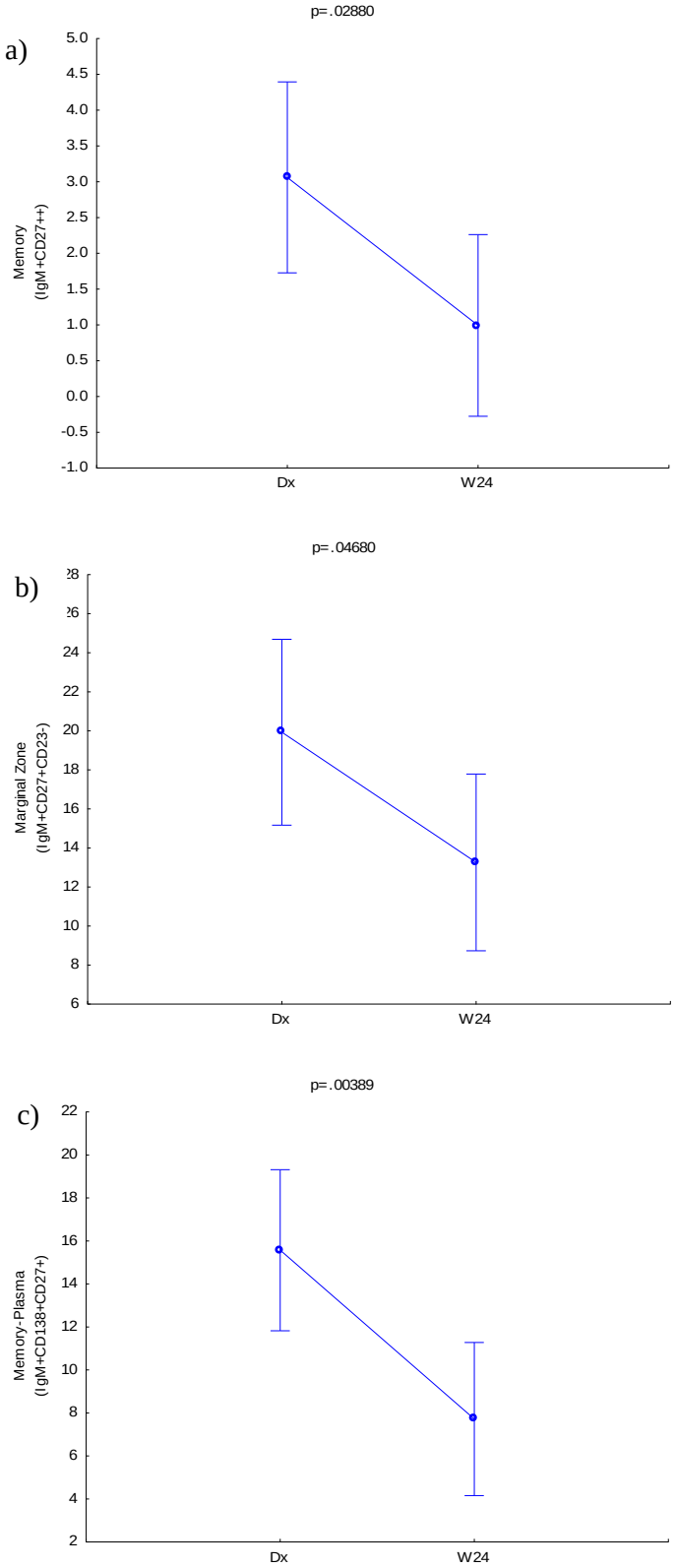


Figure 2. IgM+ B cell phenotypes that are significantly different at TB diagnosis and end of treatment (W24). A) Memory B cells, b) Marginal zone B cells and c) Memory-plasma B cells. Graphs were generated with Statitica 12 and the Mann-Whitney correction applied, based on mean values where the vertical bars denote 95% confidence. Dx = diagnosis, W24 = week 24.

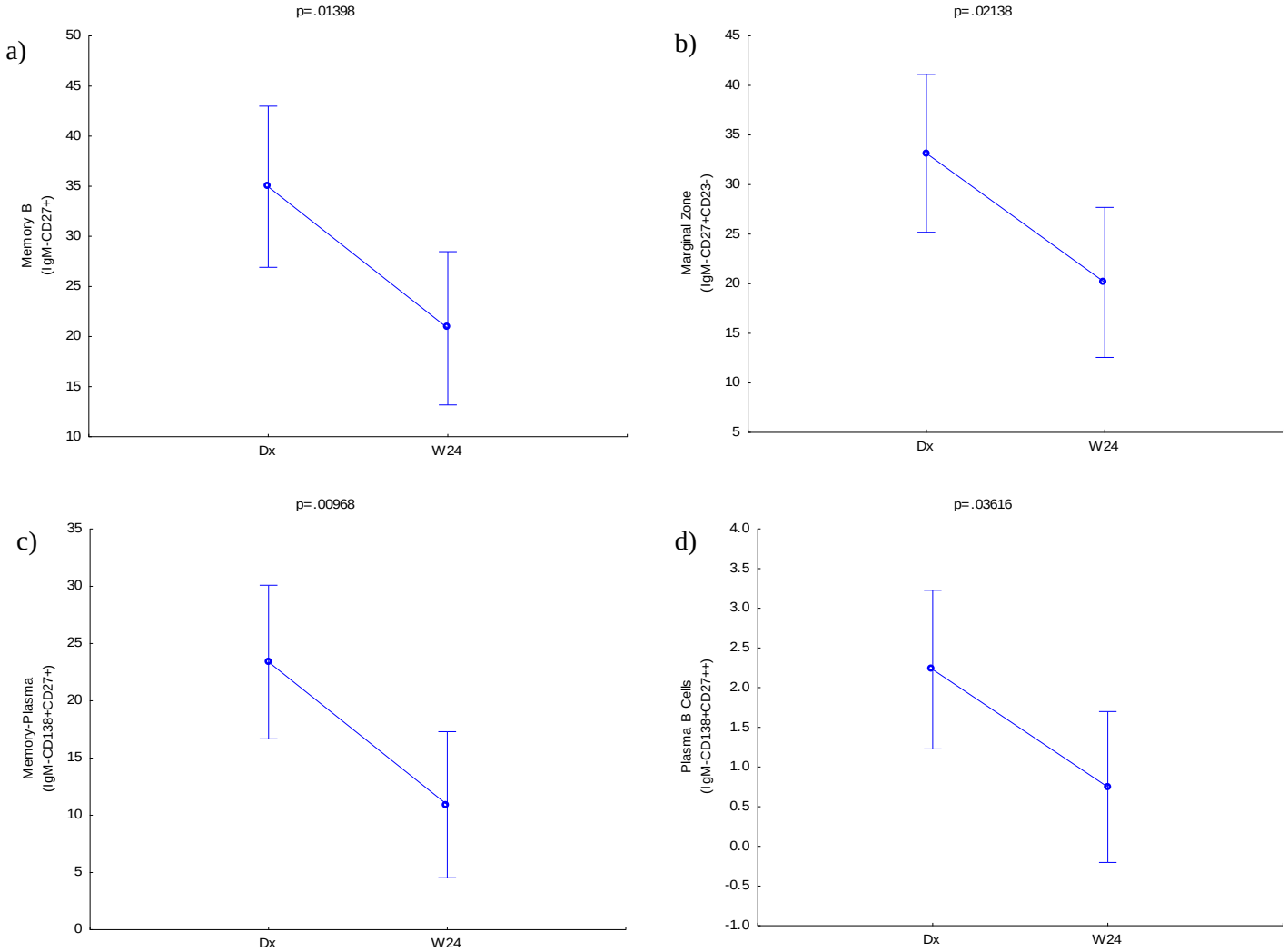
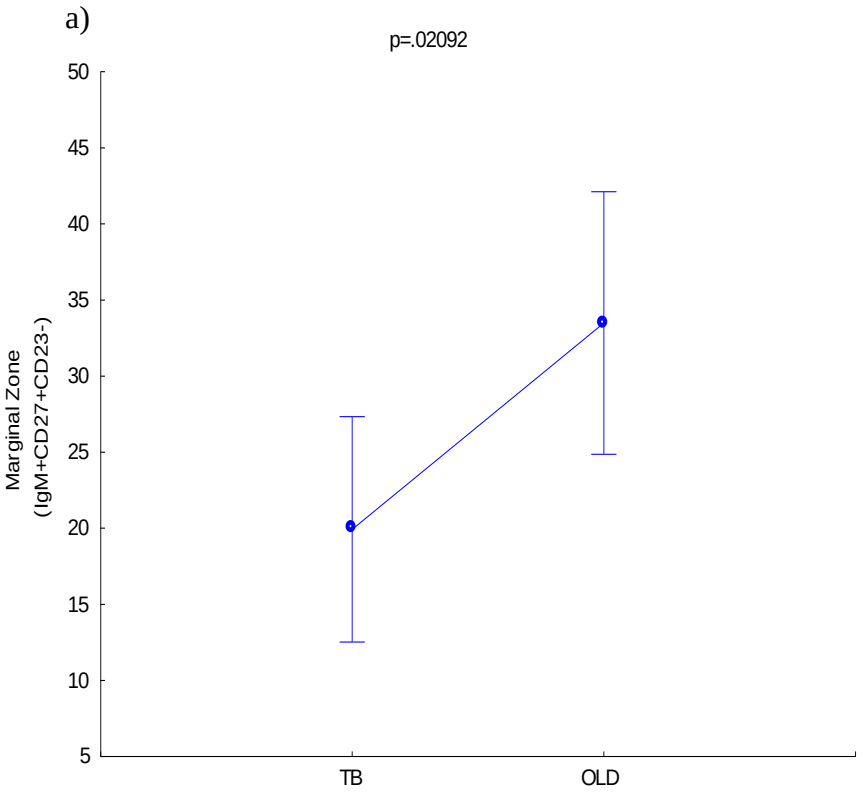
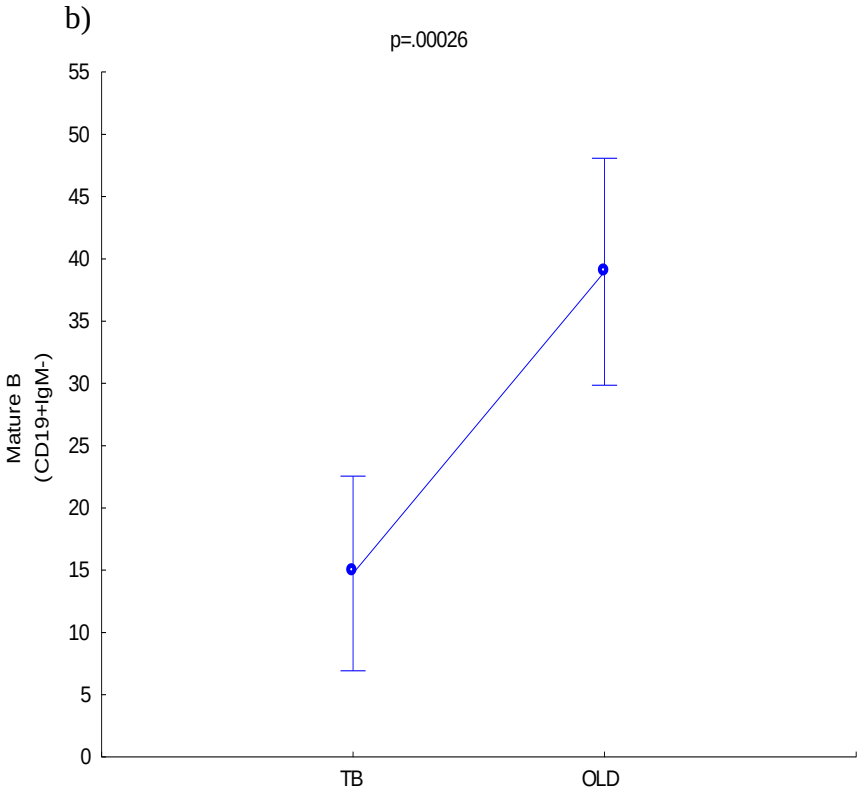


Figure 3. IgM- B cell phenotypes that are significantly different between TB diagnosis, and end of treatment (W24). a) Memory B cells, b) Marginal zone B cells, c) Memory-plasma B cells and d) Plasma B cells. Graphs were generated with Statitica 12 and the Mann-Whitney correction applied, based on mean values where the vertical bars denote 95% confidence. Dx = diagnosis, W24 = week 24





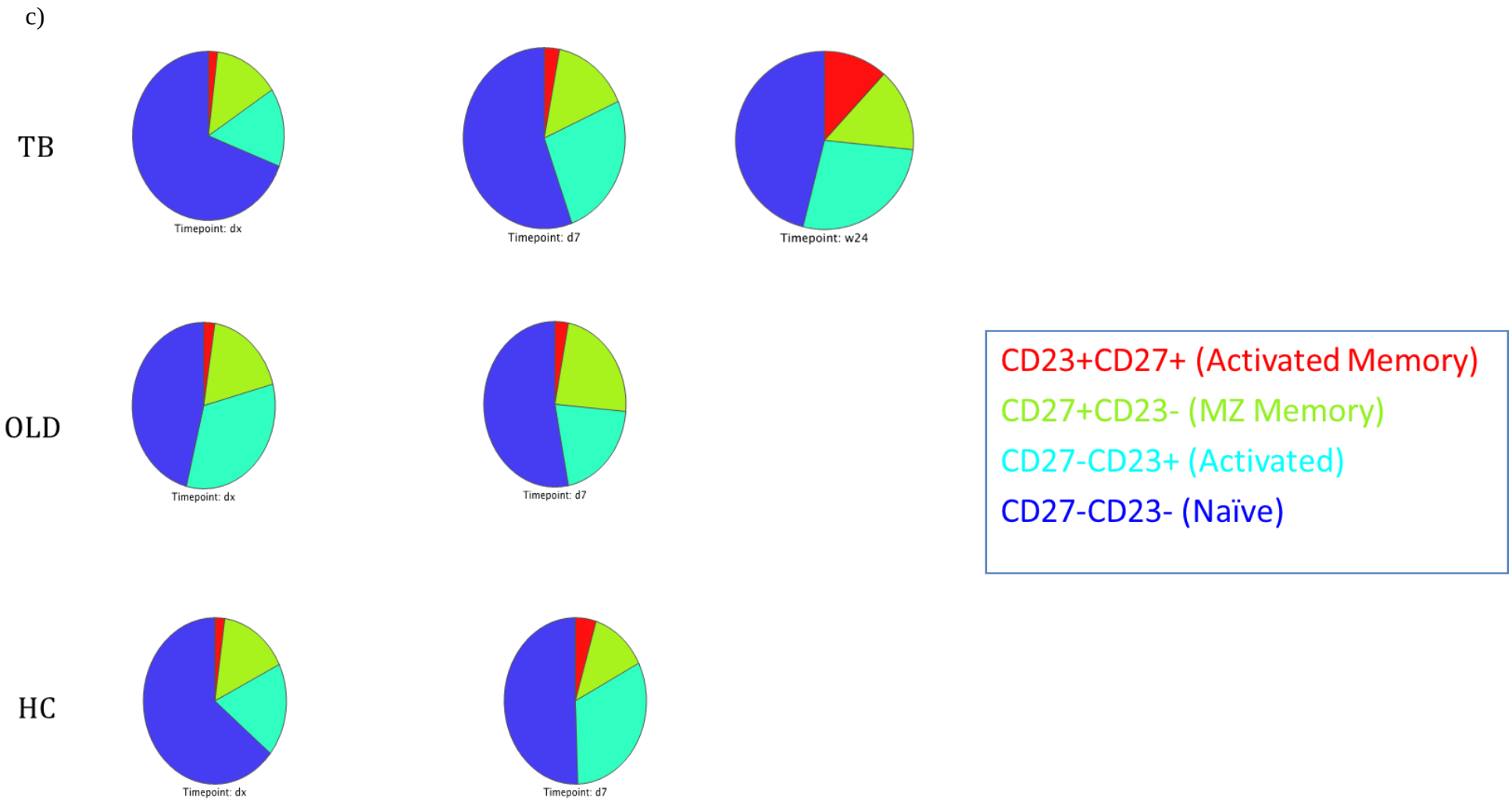


Figure 4. (a) IgM+ Marginal Zone B cells and (b) IgM- Mature B cells are significantly different between TB and OLD at diagnosis. TB = tuberculosis, OLD = other-lung disease. Graphs shown in a) and b) were generated with Statistica 12 and the Mann-Whitney correction applied, based on mean values where the vertical bars denote 95% confidence. c) Frequency distribution of Marginal Zone B cells between groups over time.

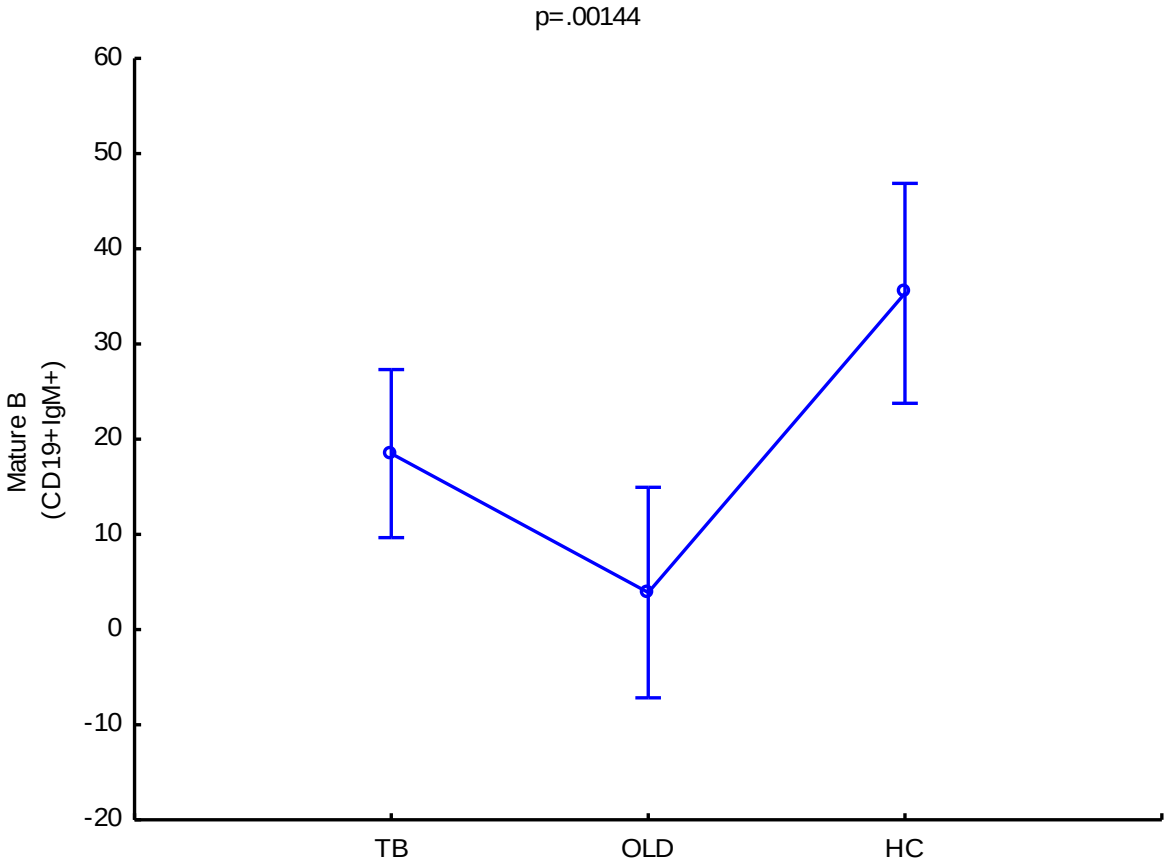


Figure 5 IgM+ Mature B cells are significantly different at diagnosis when compared to other disease states. The graph was generated with Statistica 12 and the Mann-Whitney correction applied, based on mean values with the p-values representing significant difference, where an alpha level of 95% is used. TB vs OLD: p = 0.04; TB vs HC: p = 0.02; OLD vs HC: p = 0.0003. TB = tuberculosis, OLD = other-lung disease, HC = healthy control

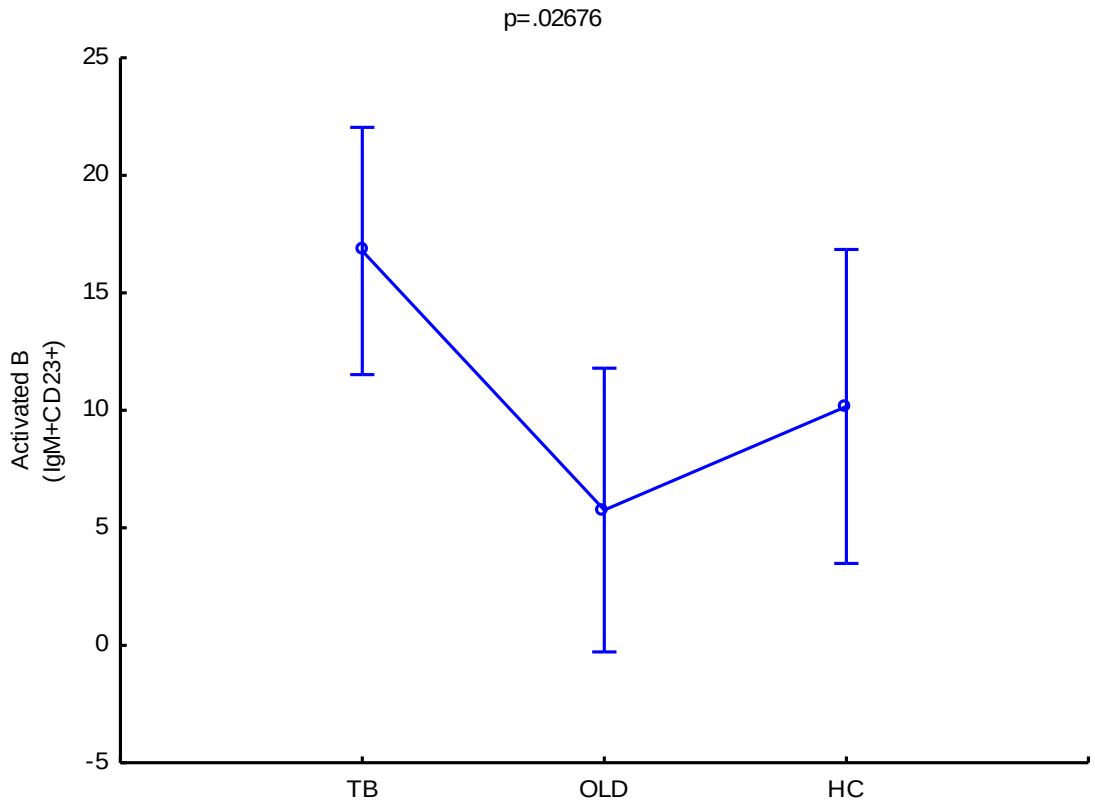


Figure 6 IgM+ Activated B cells are significantly different at diagnosis when compared to other disease states. This graph was generated with Mann-Whitney correction, based on mean values where the vertical bars denote 95% confidence. P- Values show significance between specific comparisons. TB vs OLD: p = 0.008; TB vs HC: p = 0.12; OLD vs HC: 0.32. TB = tuberculosis, OLD = other-lung disease, HC = healthy control.

Chapter 3:

The Functional Response of B cells to Antigenic Stimulation during Latent Tuberculosis

The work presented in this chapter has been formatted in the style of mBio journal to which it will be submitted for review.

The Functional Response of B cells to Antigenic Stimulation during Latent Tuberculosis

Willem J du Plessis[1], Léanie Kleynhans[1], Nelita du Plessis[1], Kim Stanley[1], Stephanus T Malherbe[1], Katharina Ronacher[1], Novel N Chegou[1], Gerhard Walzl[1] and Andre G Loxton[1]

1. SA MRC Centre for TB Research, DST/NRF Centre of Excellence for Biomedical Tuberculosis Research, Division of Molecular Biology and Human Genetics, Faculty of Medicine and Health Sciences, Stellenbosch University, Cape Town, South Africa

PO Box 241 Cape Town, 8000 South Africa

Running title: B cells during latent TB

Corresponding author:

Dr AG Loxton

Email: GL2@sun.ac.za

Tel: (+27) 21 9389399

Fax: (+27) 21 9389863

Abstract word count: 237

Text word count: 3392

Abstract

Mycobacterium tuberculosis (*M.tb*) remains a successful pathogen, causing tuberculosis disease numbers to constantly increase. Although great progress has been made in delineating the disease, the host-pathogen interaction is incompletely described. B cells have shown to function as both effectors and regulators of immunity via non-humoral pathways in both innate and adaptive immune settings. Here we assessed specific B cell functional interaction following stimulation with a broad range of antigens within the latent tuberculosis infection (LTBI) milieu. Our results indicate that B cells readily produce pro- and anti-inflammatory cytokines (including IL-1β, IL-10, IL-17, IL-21 and TNF-α) in response to stimulation. TLR4 and TLR9 based stimulations achieved the greatest secreted cytokine-production response and BCG stimulation displayed a clear preference for inducing IL-1β production. We also show that the cytokines produced by B cells are implicated strongly in cell-mediated communication and that plasma-memory B cells (CD19+CD27+CD138+) are the greatest contributors to cytokine production. Collectively our data provide insight into B cell responses, where they are implicated in and quantifies responses from specific B cell phenotypes. These findings warrant further functional B cell research with a focus on specific B cell phenotypes under conditions of active TB disease to further our knowledge about the contribution of various cell subsets which could have implications for future vaccine development or refined B cell orientated treatment in the health setting.

Introduction

Mycobacterium tuberculosis (*M.tb*) is the causative agent of tuberculosis (TB) disease and is responsible for great annual morbidity and mortality. Furthermore, the World Health Organization (WHO) reported that there were 9.6 million newly reported cases of TB coupled with 1.5 million reported deaths in 2014 (1). Although great progress has been made in delineating the disease coupled with the use of an effective chemotherapeutic regimen, TB remains endemic with the growing threat of MDR-TB (multidrug-resistant TB) and XDR-TB (extensively drug-resistant TB) (2).

A balance between regulatory and effector immune responses is in most cases sufficient to contain an *M.tb* infection to what is commonly known as latent tuberculosis infection (LTBI), as it's estimated that only 1 out of every 10 people infected with the pathogen will progress to active disease (3, 4). The lack of vaccines capable of preventing active TB places a lot of pressure on controlling the epidemic (5, 6). Numerous studies were performed in attempts to identify biomarkers not only to assist in the accurate and timely diagnosis of TB, but also to fuel the development of TB vaccines and drugs (7, 8).

The prevalence of LTBI remains high, especially in household contacts of TB patients in highly endemic settings, proving that the molecular mechanisms that constitutes and maintains the abovementioned immunologic balance between protection and/or disease progression is not well understood. Working towards understanding these mechanisms is challenging considering the overwhelming complexity observed between the biologic interaction of the host, microbe and environment (9, 10). As suggested by Barry et al, LTBI and active TB disease represents a spectrum of disease states rather than being mutually exclusive (11). This is supported by work done by Lin et al. in a cynomolgus macaque model (12). Although the factors that mediate this spectrum are not well understood, it is quite possible that the host is protected from the progression of LTBI to active TB by the enrichment of potent anti-*M.tb* specific effector cells (13).

Human tuberculosis is primarily controlled by the activation and infiltration of CD4+ Th1 cells and CD8+ cytotoxic lymphocytes (14). Although their involvement is still considered controversial (15), B cells have proven to be contribute to TB immunity in various ways.

Some of these B cell functions include presenting antigens to naïve T cells in the *M.tb* infected lung (16, 17), antibody production (18, 19) and cytokine production (20). A unique effector subset of B cells (described as innate response activator (IRA)-B cells) were identified as the primary producers of granulocyte macrophage colony-stimulating factor (GM-CSF) during experimental sepsis (21). More recently, another innate effector B cell subset was identified and implicated in the promotion of favourable Th1 responses by interferon (IFN)-γ production (22).

Taken together, it is important to explore new strategies in curbing the TB epidemic and this warrants further studies of previously underappreciated cell types in TB immunology, including B cells. We therefore focus on the non-humoral functional responses of B cells during antigenic challenge to shed light on B cell cytokine production, and the possible implications it might have on the host's immune response to *M.tb*.

Materials and Methods

Ethics statement

This study was carried out according to the Declaration of Helsinki and ethical approval was obtained for two studies utilised for sample collection from the Health Research Ethics Committee of Stellenbosch University with ethics reference #: N10/01/013 and N13/05/064.

Study subjects

For this study we recruited healthy male and female community controls between the ages of 18 and 56, who were HIV negative and with no record of previous active tuberculosis disease, were interferon gamma release assay (IGRA) (QuantiFERON TB Gold) test positive. We created a TB contact score to evaluate each participant's exposure to TB in the community. A total of eleven participants were recruited of which six (6/11, 54,5%) were male. Mean age and weight was 26±10.7 and 61.1±11.3 respectively. Heparinized whole blood (70ml) was collected at recruitment.

PBMC isolation, B cell enrichment and cryopreservation

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by using Ficoll-Paque PLUS (GE Healthcare Life Sciences density gradient centrifugation. Cell counts were performed using the Trypan Blue cell exclusion method. B cell enrichment was done using the MACS beads technology from Miltenyi Biotec through negative-selection. Enriched B cells were subsequently stored in cryo media (90% FCS and 10% DMSO) at -80˚C. The purity of the enriched B cells from PBMCs, following the MACS beads protocol, were assessed utilising anti-human CD3 (PerCP, clone UCHT1, eBioscience and anti-human CD19 (FITC, clone HIB19, eBioscience) by FACS analysis, with resulting purities above 90%.

B cell culture

Various antigens were utilised to stimulate the B cells to assess their respective responses. These antigens included: Phytohaemagglutinin (PHA, Sigma Aldrich) at 0.5mg/ml, purified protein derivative (PPD, Statens Serum Institut) at 12.5µg/ml, Lipopolysaccharides (LPS, Sigma) at 1mg/ml, Bacillus Calmette–Guérin (BCG ID Vaccine (*M. Bovis* BCG), Statens Serum Institut) at $6x10^6$ cfu/ml and the Toll-like receptor 9 agonist (TLR9a, Miltenyi Biotec) at 0.5µM. B cells were incubated at 37°C and 5% CO_2 for 16 hours. Cells were cultured in complete media consisting of RPMI (Sigma) supplemented with 10% FCS and 2mM L-Glutamine (Sigma). B cells (5×10^5cells/well) were cultured for each stimulation, in the presence of Brefeldin A (Sigma), for flow cytometry. Supernatants were collected from B cells, which were cultured in parallel without Brefeldin A, and stored at -80°C for multiplex analysis.

Multiplex cytokine Analysis

The quantification of secreted molecules in the 16-hour culture supernatants including IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13 and TNF-α was determined using the Meso Scale Discovery (MSD®) platform. Experiments were performed strictly as recommended by the manufacturer after which plates were read on a Quickplex SQ 120 instrument (MSD).

Flow cytometry

For intracellular staining, cells were cultured with Brefeldin A (Sigma) at a concentration of 10µg/ml for the duration of the stimulation. Cultured B cells were firstly stained with antibodies against cell surface markers (CD3, CD19, CD27 and CD138 – all from eBioscience) for 20 minutes, washed with FACS staining buffer (PBS, 2% FCS) and fixed and permeabilized using BD cytofix/cytoperm kit (BD Bioscience Pharmingen). These B cells were subsequently stained with antibodies against cytoplasmic proteins (IL-10, IL-17, IL-21 and TNF-α (eBioscience)) for 20 minutes in the dark and at room temperature, where after it was washed according to manufacturer's instructions (BD Bioscience). A FACS Canto II (BD Bioscience) was used for cell acquisition (≥100,000 events). The instrument was calibrated according to the manufacturer's instructions. Quality controls included the use of Rainbow Beads (eBioscience – San Diego, CA, USA) and the compensation settings were adjusted in conjunction with the use of antibody-capture beads (CompBeads, BD Biosciences) (23). Fluorescence-minus-one (FMO) control samples were utilised (as described by Perfetto *et al.* (24)) to determine appropriate gating cut-off, to increase the accuracy of distinguishing different populations.

Data and statistical analysis

Data analysis for the MSD results were done using Statistica 12, Statsoft (Ohio, USA). One-way ANOVA was done and the 95% confidence intervals plotted. Unbiased hierarchal clustering of cytokine secretion from the MSD experiments (across all stimulating conditions) and the generation of a heat map was done using Qlucore Omics Explorer (Lund, Sweden). QIAGEN's Ingenuity Pathway Analysis software (Limburg, Netherlands) was utilised to assess the involvement of differentially-expressed cytokines in immunological pathways of diseases and disorders. Wilcoxon rank sum tests, where p-value adjustment was done using the Bonferroni post-hoc test, was used for the FACS dataset. All FACS data analysis were performed in R (http://www.r-project.org).

Results

LPS and TLR9-a stimulation results in the highest cytokine-secretion upregulation.

Given the importance of cytokine mediated responses during TB, we investigated the functional capacity of B cells from latently infected individuals by stimulating naïve B cells with a broad range of antigens (from B cell-specific to TB-specific antigens) *in vitro*. The concentration of cytokines was assessed in the B cell culture supernatants after antigen stimulation using a MSD pro-inflammatory panel. In an unbiased analysis approach, Qlucore Omics Explorer software was used to generate a heat map of the antigen induced cytokine responses (figure 1). From the heat map it is clear that LPS and TLR9-a were strong inducers of pro-inflammatory markers in B cells with markers such as IL-6, IL-10 and TNF-α being upregulated. LPS stimulation further resulted in the upregulation of IL-4 whereas TLR9-a stimulation resulted in the upregulation of IL-12p70. BCG induced strong IL-1β responses and PHA strong IL-2 and IL-13 responses in B cells. When comparing B cell responses to whole blood response (in quantiferon (QFN) supernatants), an almost complete opposite was true. IL-10 and IL-6 were downregulated, in whole blood stimulated with the *M.tb* antigens ESAT-6/CFP-10/TB-7.7, TNF-α had no change in expression and IL-4 was upregulated but to a larger extent than LPS (data not shown). Furthermore, cytokines produced from these B cells are upregulated at levels which are comparable to T cell derived sources (data not shown).

B cells produce differential pro-inflammatory cytokines profiles in an antigen-dependent manner.

Univariate analysis showed that significant differences were found in six of the ten pro-inflammatory markers assessed (figure 2). It is well documented that B cells produce IL-10, and here we show that LPS and TLR9-a stimulation induce significantly higher levels, with $p \leq 0.02$ and $p \leq 0.01$ respectively, when compared to the unstimulated and the other antigens. PPD ($p = 0.024$ versus LPS), TLR9-a ($p \leq 0.01$ versus US) and LPS ($p \leq$ US, PHA and PPD) induced significantly higher concentrations of TNF-α (figure 2). The highest TNF-α concentration was observed when B cells were stimulated with LPS. A similar trend was observed with LPS inducing significantly higher concentrations of IL-6. BCG induced significantly higher concentrations of IL-1β when compared to all other stimulants ($p \leq 0.001$ versus US). BCG however did not induce IL-12p70 responses in these cells ($p \leq 0.01$ for each one respectively). PHA induced significantly higher concentrations of IL-2.

B cell IL-1β production primarily facilitates intra-cellular communication.

To follow on the over-expression of IL-1β during BCG stimulation, we analysed the respective dataset using the Ingenuity Pathway Analysis software (IPA, QIAGEN Redwood City, www.qiagen.com/ingenuity) and found that the top pathway associated was the role which cytokines play in mediating communication between immune cells (figure 3). The only cytokine from our dataset which was broadly induced was IL-8 (figure 1

and 3). What is interesting is that IL-4, IL-6 and IL-10 which are upregulated in our B cell dataset are implicated in the communication with B cells (table 1 summarizes the top five pathways B cell derived IL-1β is implicated in), with further downstream effector functions from "B effector 2 cells". These in turn produce more cytokines which are present in our dataset, suggesting a feed-forward mechanism. Our results suggest that B cell derived cytokines communicate and influence immune cells which play a key role in TB immunity. These cytokines are produced independent from the traditional T cell based sources, and could function as pro-inflammatory or anti-inflammatory effectors with possible feed-forward mechanisms, which boosts the effect.

Plasma-memory B cells are the primary source of B cell-derived cytokines

After assessing the general functional capacity of B cells, we wanted to assess in more depth which specific B cell subsets contributed the most to cytokine production following antigenic challenge. By using FACS four B cell populations were identified based on their cytokine production profile. These were defined as: plasma (PB) B cells ($CD19^+CD138^+$), mature B cells ($CD19^+CD27^-CD138^-$), memory (MB) B cells ($CD19^+CD27^+$) and plasma-memory (M-P) B cells ($CD19^+CD27^+CD138^+$). Figure 4 shows the gating strategy followed to acquire these B cell populations, and the cytokine production profiles. The graphs represented in figures 5 and 6 respectively indicates total cytokine production as contributed by each subset, where the total combined contribution adds up to 100% of production. Four cytokines, namely IL-10, IL-17, IL-21 and TNF-α, and combinations thereof were measured (figure 5). From our results it is clear that plasma-memory B cells ($CD19^+CD27^+CD138^+$) are major contributors of cytokines compared to other subsets. This is especially true for IL-10 production, where plasma-memory B cells produced significantly more ($p \leq 0.001$, and in some cases $p \leq 0.0001$) cytokines than any other subset. There seems to be a trend to cytokine production within the subsets, with the least cytokine being produced by plasma B cells ($CD19^+CD138^+$), followed by mature B cells ($CD19^+CD27^-CD138^-$) and memory B cells ($CD19^+CD27^+$) producing the second most. Interestingly, the inverse is observed when considering IL-17 production with plasma B cells being the top contributor ($p \leq 0.05$ when compared to other B cell subsets within the majority of stimulations) followed by plasma-memory cells in second place. PPD ($p = 0.0023$, compared to mature B cells) and BCG ($p = 0.0003$, compared to memory B cells) induced significantly more IL-17 than TLR9-a, which was the greatest contributing stimulant of secreted cytokine production (figures 1 and 2). The plasma-memory B cell subset also had the highest frequency of $IL\text{-}17^+IL\text{-}21^+$ cells, with $p \leq 0.001$ compared to all other populations, for both BCG and PPD (figure 6).

Discussion

It is estimated that about 2 billion individuals worldwide are infected with latent *Mycobacterium tuberculosis* (25). Although only 1 out of every 10 individuals will progress to active disease in their lifetime, people latently infected with tuberculosis still serve the purpose of acting as seedbeds for future cases of active tuberculosis disease. There has been an increase in the literature describing new functional capacities of B cells and, although great progress has been made in delineating these functions, the full functional repertoire of these cells still remains incompletely described. Here we aimed to assess the functional response of B cells to various stimuli within the milieu of latent tuberculosis infection to determine a) if they responded in a non-humoral capacity to stimulation, b) to what extent they responded and, c) if B cells respond uniquely to TB-related stimulations.

B cells displayed a significant ability to produce IL-2, IL-6, IL-10, IL-12p70, IL-1β and TNF-α following antigenic stimulations as measured from culture supernatant, with a specific preference to stimulations originating from toll-like receptors (LPS and TLR9-a) with the exception of IL-1β that was only produced in significant quantities following whole organism (BCG) stimulation.

Toll like receptors (TLRs) contain germline-encoded receptors or pattern-recognition receptors (PRR), that are pivotal during the innate recognition of pathogen associated molecular patterns (PAMPs) (26). B cells readily express a host of TLRs, including TLR4 and TLR9 (27), and although it is believed that *M.tb* is recognised by TLR1, TLR2, TLR4, TLR6, TLR8 and TLR9 (28), the majority of *M.tb* related TLR research has focussed of TLR2 and TLR4. Several groups have shown that polymorphisms in TLRs are associated with increased susceptibility to tuberculosis (28–31). TLRs are not only implicated in TB disease susceptibility, but also crucial for host protection as a study on $TLR4^{-/-}$ mice resulted in exacerbated and disseminated disease coupled with neutrophilia, reduced macrophage recruitment and poor outcome (32). The findings of our study highlights the significance of TLR responses not only for the possible influence they might have within TB disease, but the powerful link they fulfil between innate and adaptive responses by activating B cells on an innate level which results in intrinsic adaptive responses from the B cells with subsequent cytokine production.

While TLRs and C-type lectin receptors are responsible for recognising extracellular mycobacterial components, aryl hydrocarbon receptors (AhRs) and nucleotide-binding oligomerization domain-like receptors (NLRs) sense, and readily respond to mycobacterial molecules found intracellularly in the cytosol (33, 34). The resulting signalling cascades from these receptors facilitate the production of pro-inflammatory molecules such as cytokines, chemokines and anti-microbial molecules that control cell death (35). These include cytokines which have proven to be critical in the host response against tuberculosis, including IL-12, TNF-α and IL-1β (36). IL-1β is intricately connected to the inflammasome, which is commonly found in innate cells and functions as molecular platforms from which pro-inflammatory cytokines (such as IL-1β) can mature following activation caused by cellular- stresses and infection (37). Inflammasomes are common amongst innate cells such as dendritic cells and macrophages (38), but whether they can be activated and function from within lymphocytes (such as B cells) remains unclear. Inflammasomes are, however, implicated in the host

immune response against *M.tb* (39–42) and have recently been the target of a promising new TB vaccine that utilises recombinant BCG (43). Our data shows that B cell stimulation with BCG, and not PPD, resulted in the significant production of IL-1β. This finding raises the question as to the importance of whole-bacterium presence in the successful activation of the inflammasome platform during mycobacterial challenge.

Although a lot of work has been done in identifying both the pro- and anti-inflammatory capacity of B cells (20, 44–47), the knowledge regarding which specific phenotypic subsets of B cells are responsible for the production of specific cytokines is incompletely described. Here we aimed to not only demonstrate B cell cytokine production following specific stimulation, but to shed light on the phenotype of the major cytokine producing subsets. Several studies have focussed on the regulatory functions of B cells (those producing IL-10 and IL-35) and subsequently showed that antibody secreting B cells with a $CD19^{+}CD138^{hi}$ phenotype were the primary producers of IL-10 and IL-35 (44, 48, 49). Here we show for the first time that IL-10 is produced in significantly greater quantities by $CD19^{+}CD27^{+}CD138^{+}$ B cells when compared to $CD19^{+}CD138^{+}$ B cells, regardless of stimulating condition. This finding places new emphasis on accurately distinguishing specific B cell subsets when assessing function and possible downstream effects. We further demonstrated that plasma-memory B cells is also the drivers of TNF-α and IL-21 production. TNF-α is produced by many cell types and has shown to have good cytotoxic synergy with human interferon (50), where TNF-α is required for protective immunity in mice (51). Plasma cells are inherently short lived, but the effects of TNF-α on these cells suggests that their lifetime can be extended (52). TNF-α is also required for the formation and maintenance of granulomas and can even influence the production of chemokines during *M.tb* challenge (53–56). IL-21 is a cytokine with pleiotropic effects, which includes the differentiation of naïve- and memory B cells into plasma cells, as well as having the capacity to induce the maturity of $CD8^{+}$ T cells with enhanced cytotoxicity (57). IL-21R was shown to be expressed on germinal centre and naïve B cells, but not on memory- or plasma B cells (58). Interestingly, we found that the frequency of B cell derived IL-21 was the greatest when compared to the other cytokines in our dataset (data not shown). This would suggest that B cells, and specifically memory- or plasma B cells, regulate the activation and maturation of more B cells *via* an autocrine loop following antigenic stimulation. Another interesting finding was that IL-17 was predominantly produced by plasma B cells ($CD19^{+}CD138^{+}$), and not plasma-memory B cells like the other cytokines. These findings are in accordance with Bermejo *et al.* (59). The observed IL-17 production from B cells could be activated independently from the usual major requirements for IL-17 production. This included IL-6, IL-23, AhR, RoRgt and RORa for T cells; and independent of MyD88 and CD40, who are both major pathways of activation in B cells (59). Our findings reinforce the notion that B cells could function in the innate control of *M.tb via* IL-17 production.

Our study suggests that B cells readily respond to a host of stimulations in a non-humoral manner. These findings warrant further research into the functional capacity of B cells within the tuberculosis milieu, with a focus on specific phenotypes and cell subsets. The identification of B cell subsets or features that functionally produce pro-inflammatory and/or anti-inflammatory cytokines could pave the way for new strategies regarding vaccine development and B cell directed treatment in the clinic. For example, the selective depletion of B cell

subsets implicated in disease pathology without affecting the remaining B cell subsets producing cytokines associated with disease resolution would be of great benefit to the patient.

Acknowledgements

A special thank you to the participants and field staff of the Immunology Research Group (SUNIRG). WJ du Plessis holds an NRF scholarship. GW holds the DST/NRF SA Research Chair Initiative (SARChI) for TB Biomarkers. The authors declare no conflict of interest.

References

1. **WHO**. 2015. Global Tuberculosis Report 2015. World Heal Organ.

2. **Abubakar I**, **Zignol M**, **Falzon D**, **Raviglione M**, **Ditiu L**, **Masham S**, **Adetifa I**, **Ford N**, **Cox H**, **Lawn SD**, **Marais BJ**, **McHugh TD**, **Mwaba P**, **Bates M**, **Lipman M**, **Zijenah L**, **Logan S**, **McNerney R**, **Zumla A**, **Sarda K**, **Nahid P**, **Hoelscher M**, **Pletschette M**, **Memish ZA**, **Kim P**, **Hafner R**, **Cole S**, **Migliori GB**, **Maeurer M**, **Schito M**, **Zumla A**. 2013. Drug-resistant tuberculosis: time for visionary political leadership. Lancet Infect Dis **13**:529–539.

3. **Comstock GW**, **Livesay VT**, **Woolpert SF**. 1974. The prognosis of a positive tuberculin reaction in childhood and adolescence. Am J Epidemiol **99**:131–8.

4. **Vynnycky E**, **Fine PE**. 2000. Lifetime risks, incubation period, and serial interval of tuberculosis. Am J Epidemiol **152**:247–63.

5. **Kaufmann SHE**. 2012. Tuberculosis vaccine development: strength lies in tenacity. Trends Immunol **33**:373–9.

6. **McShane H**, **Jacobs WR**, **Fine PE**, **Reed SG**, **McMurray DN**, **Behr M**, **Williams A**, **Orme IM**. 2012. BCG: myths, realities, and the need for alternative vaccine strategies. Tuberculosis (Edinb) **92**:283–8.

7. **Walzl G**, **Ronacher K**, **Hanekom W**, **Scriba TJ**, **Zumla A**. 2011. Immunological biomarkers of tuberculosis. Nat Rev Immunol **11**:343–54.

8. **Ottenhoff THM**, **Kaufmann SHE**. 2012. Vaccines against tuberculosis: where are we and where do we need to go? PLoS Pathog **8**:e1002607.

9. **Lebeis SL**, **Kalman D**. 2009. Aligning antimicrobial drug discovery with complex and redundant host-pathogen interactions. Cell Host Microbe **5**:114–22.

10. **Lenaerts A**, **Barry CE**, **Dartois V**. 2015. Heterogeneity in tuberculosis pathology, microenvironments and therapeutic responses. Immunol Rev **264**:288–307.

11. **Barry CE**, **Boshoff HI**, **Dartois V**, **Dick T**, **Ehrt S**, **Flynn J**, **Schnappinger D**, **Wilkinson RJ**, **Young D**. 2009. The spectrum of latent tuberculosis: rethinking the biology and intervention strategies. Nat Rev Microbiol **7**:845–55.

12. **Lin PL**, **Rodgers M**, **Smith L**, **Bigbee M**, **Myers A**, **Bigbee C**, **Chiosea I**, **Capuano S V.**, **Fuhrman C**, **Klein E**, **Flynn JL**. 2009. Quantitative Comparison of Active and Latent Tuberculosis in the Cynomolgus Macaque Model. Infect Immun **77**:4631–4642.

13. **Rao M**, **Valentini D**, **Poiret T**, **Dodoo E**, **Parida S**, **Zumla A**, **Brighenti S**, **Maeurer M**. 2015. B in TB: B Cells as Mediators of Clinically Relevant Immune Responses in Tuberculosis. Clin Infect Dis **61Suppl 3**:S225–34.

14. **Brighenti S**, **Andersson J**. 2012. Local Immune Responses in Human Tuberculosis: Learning From the Site of Infection. J Infect Dis **205**:S316–S324.

15. **Achkar JM**, **Chan J**, **Casadevall A**. 2015. Role of B cells and antibodies in acquired immunity against Mycobacterium tuberculosis. Cold Spring Harb Perspect Med **5**:a018432.

16. **Harris DP**, **Haynes L**, **Sayles PC**, **Duso DK**, **Eaton SM**, **Lepak NM**, **Johnson LL**, **Swain SL**, **Lund FE**. 2000. Reciprocal regulation of polarized cytokine production by effector B and T cells. Nat Immunol **1**:475–82.

17. **de Wit J**, **Jorritsma T**, **Makuch M**, **Remmerswaal EBM**, **Klaasse Bos H**, **Souwer Y**, **Neefjes J**, **Ten Berge IJM**, **van Ham SM**. 2014. Human B cells promote T-cell plasticity to optimize antibody response by inducing coexpression of TH1/TFH signatures. J Allergy Clin Immunol.

18. **Hoehlig K**, **Lampropoulou V**, **Roch T**, **Neves P**, **Calderon-Gomez E**, **Anderton SM**, **Steinhoff U**, **Fillatreau S**. 2008. Immune regulation by B cells and antibodies a view towards the clinic. Adv Immunol **98**:1–38.

19. **Chan J**, **Mehta S**, **Bharrhan S**, **Chen Y**, **Achkar JM**, **Casadevall A**, **Flynn J**. 2014. The role

of B cells and humoral immunity in Mycobacterium tuberculosis infection. Semin Immunol **26**:588–600.

20. **Lund FE**. 2008. Cytokine-producing B lymphocytes-key regulators of immunity. Curr Opin Immunol **20**:332–8.

21. **Rauch PJ**, **Chudnovskiy A**, **Robbins CS**, **Weber GF**, **Etzrodt M**, **Hilgendorf I**, **Tiglao E**, **Figueiredo J-L**, **Iwamoto Y**, **Theurl I**, **Gorbatov R**, **Waring MT**, **Chicoine AT**, **Mouded M**, **Pittet MJ**, **Nahrendorf M**, **Weissleder R**, **Swirski FK**. 2012. Innate response activator B cells protect against microbial sepsis. Science **335**:597–601.

22. **Bao Y**, **Liu X**, **Han C**, **Xu S**, **Xie B**, **Zhang Q**, **Gu Y**, **Hou J**, **Qian L**, **Qian C**, **Han H**, **Cao X**. 2013. Identification of IFN-γ-producing innate B cells. Cell Res **24**:161–176.

23. **Baumgarth N**, **Roederer M**. 2000. A practical approach to multicolor flow cytometry for immunophenotyping. J Immunol Methods **243**:77–97.

24. **Perfetto SP**, **Chattopadhyay PK**, **Roederer M**. 2004. Seventeen-colour flow cytometry: unravelling the immune system. Nat Rev Immunol **4**:648–55.

25. **Rangaka MX**, **Cavalcante SC**, **Marais BJ**, **Thim S**, **Martinson NA**, **Swaminathan S**, **Chaisson RE**. 2015. Controlling the seedbeds of tuberculosis: diagnosis and treatment of tuberculosis infection. Lancet (London, England) **6736**.

26. **Akira S**, **Takeda K**, **Kaisho T**. 2001. Toll-like receptors: critical proteins linking innate and acquired immunity. Nat Immunol **2**:675–80.

27. **Hornung V**, **Rothenfusser S**, **Britsch S**, **Krug A**, **Jahrsdörfer B**, **Giese T**, **Endres S**, **Hartmann G**. 2002. Quantitative expression of toll-like receptor 1-10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides. J Immunol **168**:4531–7.

28. **Zhao L**, **Liu K**, **Kong X**, **Tao Z**, **Wang Y**, **Liu Y**. 2015. Association of polymorphisms in

Toll-like receptors 4 and 9 with risk of pulmonary tuberculosis: a meta-analysis. Med Sci Monit **21**:1097–106.

29. **Salie M**, **Daya M**, **Lucas LA**, **Warren RM**, **van der Spuy GD**, **van Helden PD**, **Hoal EG**, **Möller M**. 2015. Association of toll-like receptors with susceptibility to tuberculosis suggests sex-specific effects of TLR8 polymorphisms. Infect Genet Evol **34**:221–9.

30. **Schurz H**, **Daya M**, **Möller M**, **Hoal EG**, **Salie M**. 2015. TLR1, 2, 4, 6 and 9 Variants Associated with Tuberculosis Susceptibility: A Systematic Review and Meta-Analysis. PLoS One **10**:e0139711.

31. **Wu L**, **Hu Y**, **Li D**, **Jiang W**, **Xu B**. 2015. Screening toll-like receptor markers to predict latent tuberculosis infection and subsequent tuberculosis disease in a Chinese population. BMC Med Genet **16**:19.

32. **Abel B**, **Thieblemont N**, **Quesniaux VJF**, **Brown N**, **Mpagi J**, **Miyake K**, **Bihl F**, **Ryffel B**. 2002. Toll-like receptor 4 expression is required to control chronic Mycobacterium tuberculosis infection in mice. J Immunol **169**:3155–62.

33. **Killick KE**, **Ní Cheallaigh C**, **O'Farrelly C**, **Hokamp K**, **MacHugh DE**, **Harris J**. 2013. Receptor-mediated recognition of mycobacterial pathogens. Cell Microbiol **15**:1484–95.

34. **Moura-Alves P**, **Faé K**, **Houthuys E**, **Dorhoi A**, **Kreuchwig A**, **Furkert J**, **Barison N**, **Diehl A**, **Munder A**, **Constant P**, **Skrahina T**, **Guhlich-Bornhof U**, **Klemm M**, **Koehler A-B**, **Bandermann S**, **Goosmann C**, **Mollenkopf H-J**, **Hurwitz R**, **Brinkmann V**, **Fillatreau S**, **Daffe M**, **Tümmler B**, **Kolbe M**, **Oschkinat H**, **Krause G**, **Kaufmann SHE**. 2014. AhR sensing of bacterial pigments regulates antibacterial defence. Nature **512**:387–92.

35. **Kaufmann SHE**, **Dorhoi A**. 2013. Inflammation in tuberculosis: interactions, imbalances and interventions. Curr Opin Immunol **25**:441–9.

36. **Cooper AM**, **Mayer-Barber KD**, **Sher A**. 2011. Role of innate cytokines in mycobacterial

infection. Mucosal Immunol **4**:252–260.

37. **Schroder K**, **Tschopp JJJ**. 2010. The inflammasomes. Cell **140**:821–32.

38. **Briken V**, **Ahlbrand SE**, **Shah S**. 2013. Mycobacterium tuberculosis and the host cell inflammasome: a complex relationship. Front Cell Infect Microbiol **3**:62.

39. **Zhou Y**, **Zhao D**, **Yue R**, **Khan SH**, **Shah SZA**, **Yin X**, **Yang L**, **Zhang Z**, **Zhou X**. 2015. Inflammasomes-dependent regulation of IL-1β secretion induced by the virulent Mycobacterium bovis Beijing strain in THP-1 macrophages. Antonie Van Leeuwenhoek **108**:163–71.

40. **Mayer-Barber KD**, **Andrade BB**, **Barber DL**, **Hieny S**, **Feng CG**, **Caspar P**, **Oland S**, **Gordon S**, **Sher A**. 2011. Innate and adaptive interferons suppress IL-1α and IL-1β production by distinct pulmonary myeloid subsets during Mycobacterium tuberculosis infection. Immunity **35**:1023–34.

41. **Mayer-Barber KD**, **Barber DL**, **Shenderov K**, **White SD**, **Wilson MS**, **Cheever A**, **Kugler D**, **Hieny S**, **Caspar P**, **Núñez G**, **Schlueter D**, **Flavell RA**, **Sutterwala FS**, **Sher A**. 2010. Caspase-1 independent IL-1beta production is critical for host resistance to mycobacterium tuberculosis and does not require TLR signaling in vivo. J Immunol **184**:3326–30.

42. **McElvania Tekippe E**, **Allen IC**, **Hulseberg PD**, **Sullivan JT**, **McCann JR**, **Sandor M**, **Braunstein M**, **Ting JP-Y**. 2010. Granuloma formation and host defense in chronic Mycobacterium tuberculosis infection requires PYCARD/ASC but not NLRP3 or caspase-1. PLoS One **5**:e12320.

43. **Saiga H**, **Nieuwenhuizen N**, **Gengenbacher M**, **Koehler A-B**, **Schuerer S**, **Moura-Alves P**, **Wagner I**, **Mollenkopf H-J**, **Dorhoi A**, **Kaufmann SHE**. 2015. The Recombinant BCG ΔureC::hly Vaccine Targets the AIM2 Inflammasome to Induce Autophagy and Inflammation. J Infect Dis **211**:1831–41.

44. **Shen P**, **Roch T**, **Lampropoulou V**, **O'Connor RA**, **Stervbo U**, **Hilgenberg E**, **Ries S**, **Dang VD**, **Jaimes Y**, **Daridon C**, **Li R**, **Jouneau L**, **Boudinot P**, **Wilantri S**, **Sakwa I**, **Miyazaki Y**, **Leech MD**, **McPherson RC**, **Wirtz S**, **Neurath M**, **Hoehlig K**, **Meinl E**, **Grützkau A**, **Grün JR**, **Horn K**, **Kühl AA**, **Dörner T**, **Bar-Or A**, **Kaufmann SHE**, **Anderton SM**, **Fillatreau S**. 2014. IL-35-producing B cells are critical regulators of immunity during autoimmune and infectious diseases. Nature **507**:366–70.

45. **Barr TA**, **Shen P**, **Brown S**, **Lampropoulou V**, **Roch T**, **Lawrie S**, **Fan B**, **O'Connor RA**, **Anderton SM**, **Bar-Or A**, **Fillatreau S**, **Gray D**. 2012. B cell depletion therapy ameliorates autoimmune disease through ablation of IL-6-producing B cells. J Exp Med **209**:1001–10.

46. **Fernando TR**, **Rodriguez-Malave NI**, **Rao DS**. 2012. MicroRNAs in B cell development and malignancy. J Hematol Oncol **5**:7.

47. **Fillatreau S**, **Sweenie CH**, **McGeachy MJ**, **Gray D**, **Anderton SM**. 2002. B cells regulate autoimmunity by provision of IL-10. Nat Immunol **3**:944–50.

48. **Matsumoto M**, **Baba A**, **Yokota T**, **Nishikawa H**, **Ohkawa Y**, **Kayama H**, **Kallies A**, **Nutt SL**, **Sakaguchi S**, **Takeda K**, **Kurosaki T**, **Baba Y**. 2014. Interleukin-10-Producing Plasmablasts Exert Regulatory Function in Autoimmune Inflammation. Immunity **41**:1040–1051.

49. **Lampropoulou V**, **Hoehlig K**, **Roch T**, **Neves P**, **Calderón Gómez E**, **Sweenie CH**, **Hao Y**, **Freitas AA**, **Steinhoff U**, **Anderton SM**, **Fillatreau S**. 2008. TLR-activated B cells suppress T cell-mediated autoimmunity. J Immunol **180**:4763–73.

50. **Williamson BD**, **Carswell EA**, **Rubin BY**, **Prendergast JS**, **Old LJ**. 1983. Human tumor necrosis factor produced by human B-cell lines: synergistic cytotoxic interaction with human interferon. Proc Natl Acad Sci U S A **80**:5397–401.

51. **Flynn JL**, **Goldstein MM**, **Chan J**, **Triebold KJ**, **Pfeffer K**, **Lowenstein CJ**, **Schhreiber R**,

Mak TW, **Bloom BR**. 1995. Tumor necrosis factor-alpha is required in the protective immune response against mycobacterium tuberculosis in mice. Immunity **2**:561–572.

52. **Cassese G**, **Arce S**, **Hauser AE**, **Lehnert K**, **Moewes B**, **Mostarac M**, **Muehlinghaus G**, **Szyska M**, **Radbruch A**, **Manz RA**. 2003. Plasma Cell Survival Is Mediated by Synergistic Effects of Cytokines and Adhesion-Dependent Signals. J Immunol **171**:1684–1690.

53. **Mohan VP**, **Scanga CA**, **Yu K**, **Scott HM**, **Tanaka KE**, **Tsang E**, **Tsai MM**, **Flynn JL**, **Chan J**. 2001. Effects of tumor necrosis factor alpha on host immune response in chronic persistent tuberculosis: possible role for limiting pathology. Infect Immun **69**:1847–55.

54. **Algood HMS**, **Lin PL**, **Yankura D**, **Jones A**, **Chan J**, **Flynn JL**. 2004. TNF influences chemokine expression of macrophages in vitro and that of CD11b+ cells in vivo during Mycobacterium tuberculosis infection. J Immunol **172**:6846–57.

55. **Bean AG**, **Roach DR**, **Briscoe H**, **France MP**, **Korner H**, **Sedgwick JD**, **Britton WJ**. 1999. Structural deficiencies in granuloma formation in TNF gene-targeted mice underlie the heightened susceptibility to aerosol Mycobacterium tuberculosis infection, which is not compensated for by lymphotoxin. J Immunol **162**:3504–11.

56. **Keane J**, **Gershon S**, **Wise RP**, **Mirabile-Levens E**, **Kasznica J**, **Schwieterman WD**, **Siegel JN**, **Braun MM**. 2001. Tuberculosis associated with infliximab, a tumor necrosis factor alpha-neutralizing agent. N Engl J Med **345**:1098–104.

57. **Gharibi T**, **Majidi J**, **Kazemi T**, **Dehghanzadeh R**, **Motallebnezhad M**, **Babaloo Z**. 2015. Biological effects of IL-21 on different immune cells and its role in autoimmune diseases. Immunobiology.

58. **Good KL**, **Bryant VL**, **Tangye SG**. 2006. Kinetics of human B cell behavior and amplification of proliferative responses following stimulation with IL-21. J Immunol **177**:5236–47.

59. **Bermejo DA**, **Jackson SW**, **Gorosito-Serran M**, **Acosta-Rodriguez E V**, **Amezcua-Vesely MC**, **Sather BD**, **Singh AK**, **Khim S**, **Mucci J**, **Liggitt D**, **Campetella O**, **Oukka M**, **Gruppi A**, **Rawlings DJ**. 2013. Trypanosoma cruzi trans-sialidase initiates a program independent of the transcription factors RORγt and Ahr that leads to IL-17 production by activated B cells. Nat Immunol **14**:514–22.

Figures and Tables

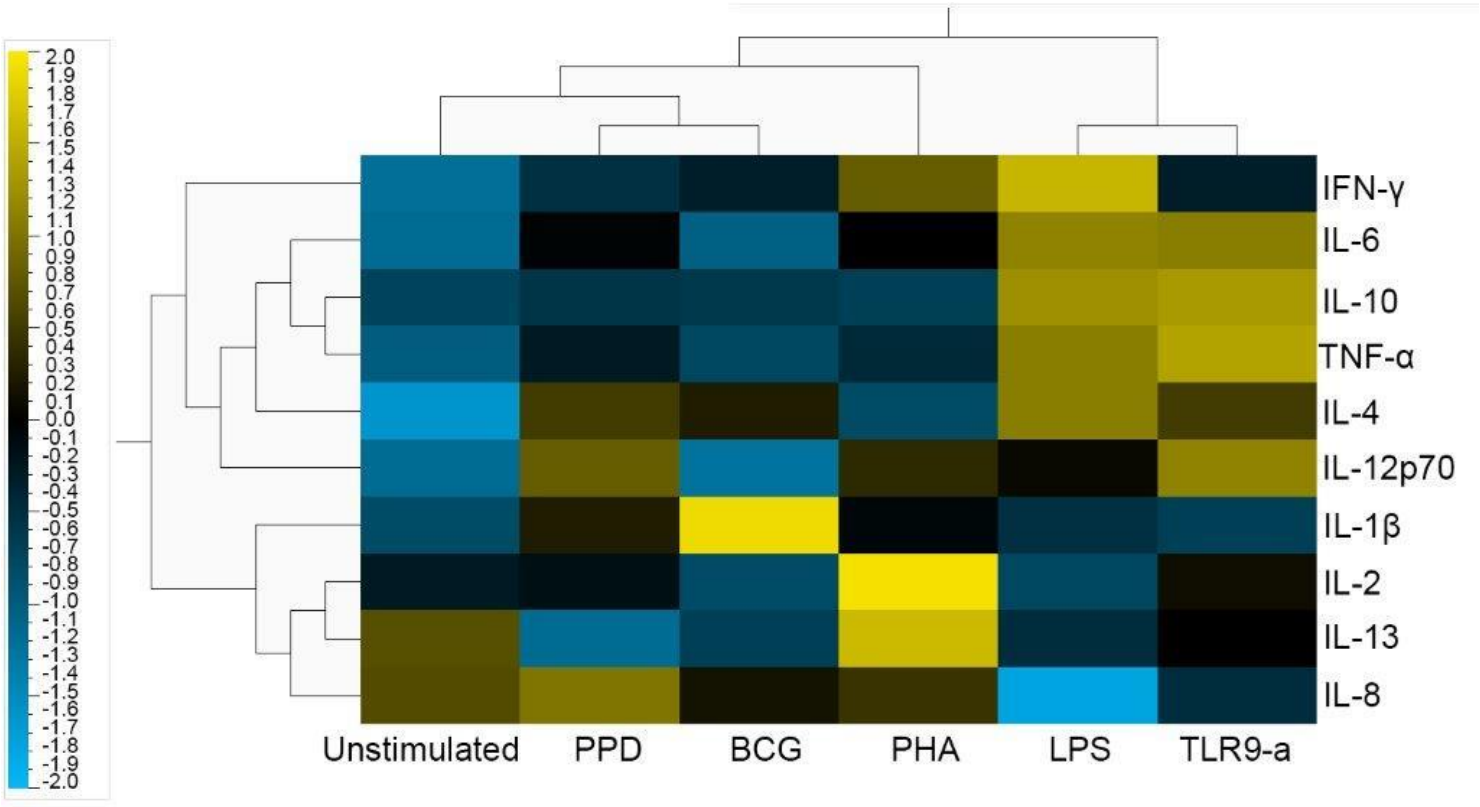


Figure 1. Differential secretion of cytokines in B cell supernatant following a 16-hour stimulation with multiple antigens. Qlucore Omics explorer software was used to do an unbiased hierarchical analysis and generate a heat map where cytokines were clustered based on expression within each stimulating condition. Green represents cytokines under-expressed while red represents cytokines being over-expressed. The graph represents mean values from 11 samples.

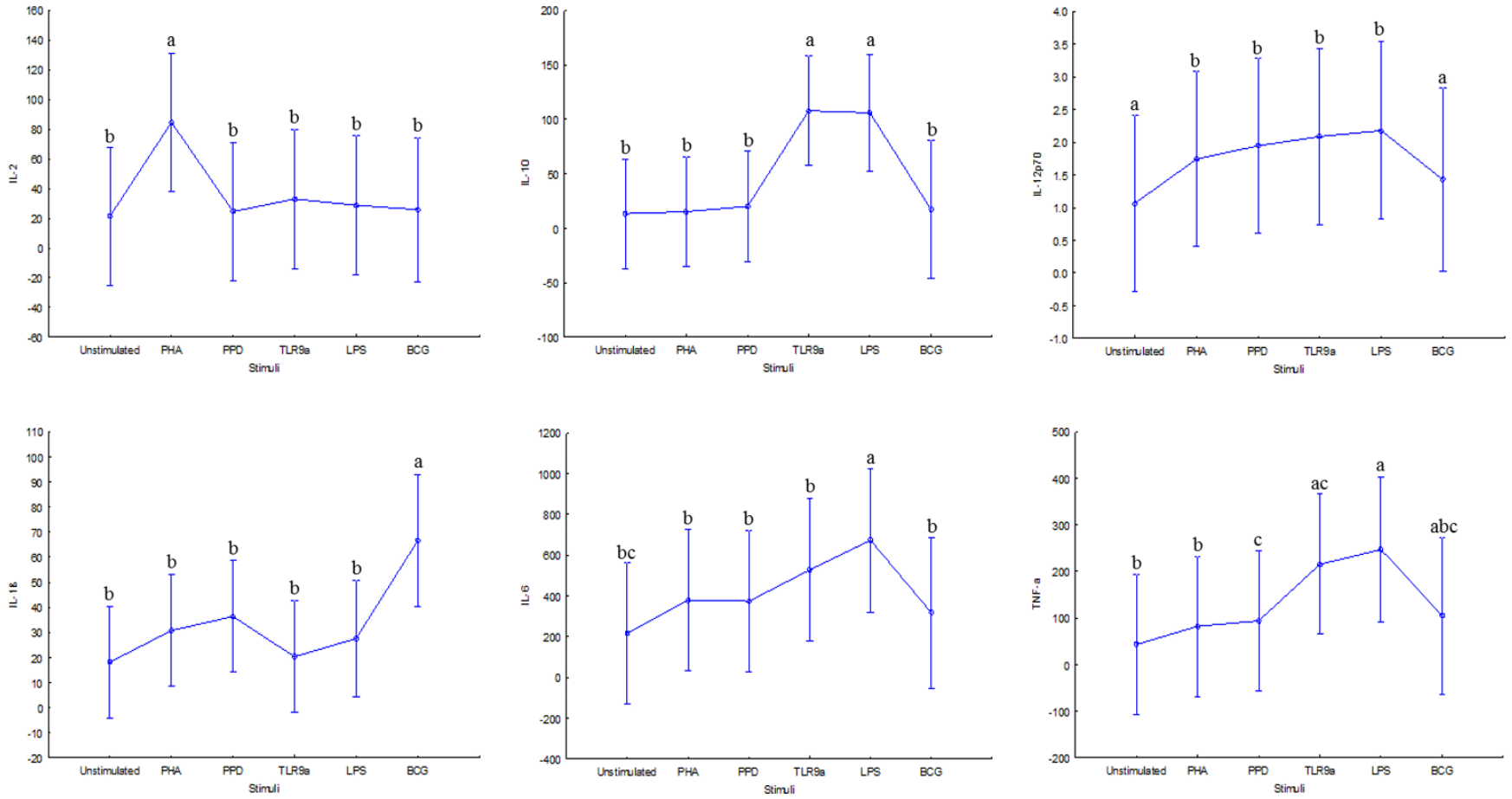


Figure 2. B cells produce pro-inflammatory cytokines differentially based on stimulation. B cells were stimulated for 16 hours in the presence of various antigen before the supernatant was analysed on the MSD platform to quantify specific pro-inflammatory cytokine. Points on the graph with commonly shared letters indicate no significant difference between those points. A confidence interval of 95% is depicted.

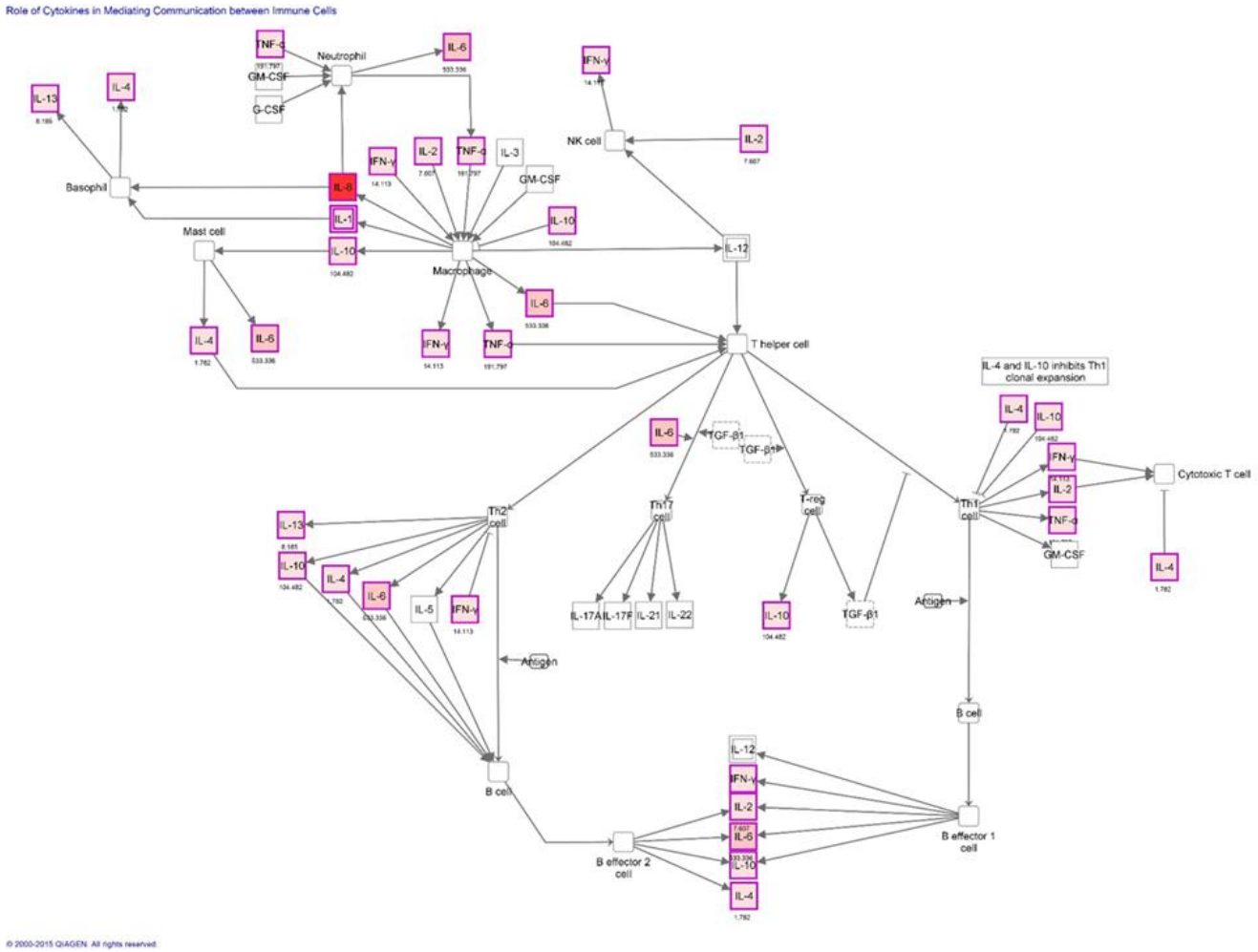


Figure 3. B cell cytokine production is primarily implicated in mediating communication between immune cells. Ingenuity pathway analysis (IPA) software indicated that the upregulation of IL-1β following BCG stimulation are primarily implicated in pathways which facilitate cellular communication between immune cells, with a p-value of 5,83E−11. The second top pathway it's implicated in is T-helper cell differentiation with a p-value of 1,65E−10, and is also implicated in the communication between innate and adaptive immune cells with a p-value of 3,77E−10. Purple boxes represent markers that are from the dataset and participate in the pathway. Red boxes indicate markers present in the dataset that are upregulated in the pathway. White boxes indicate markers present in the pathway, but not in the dataset.

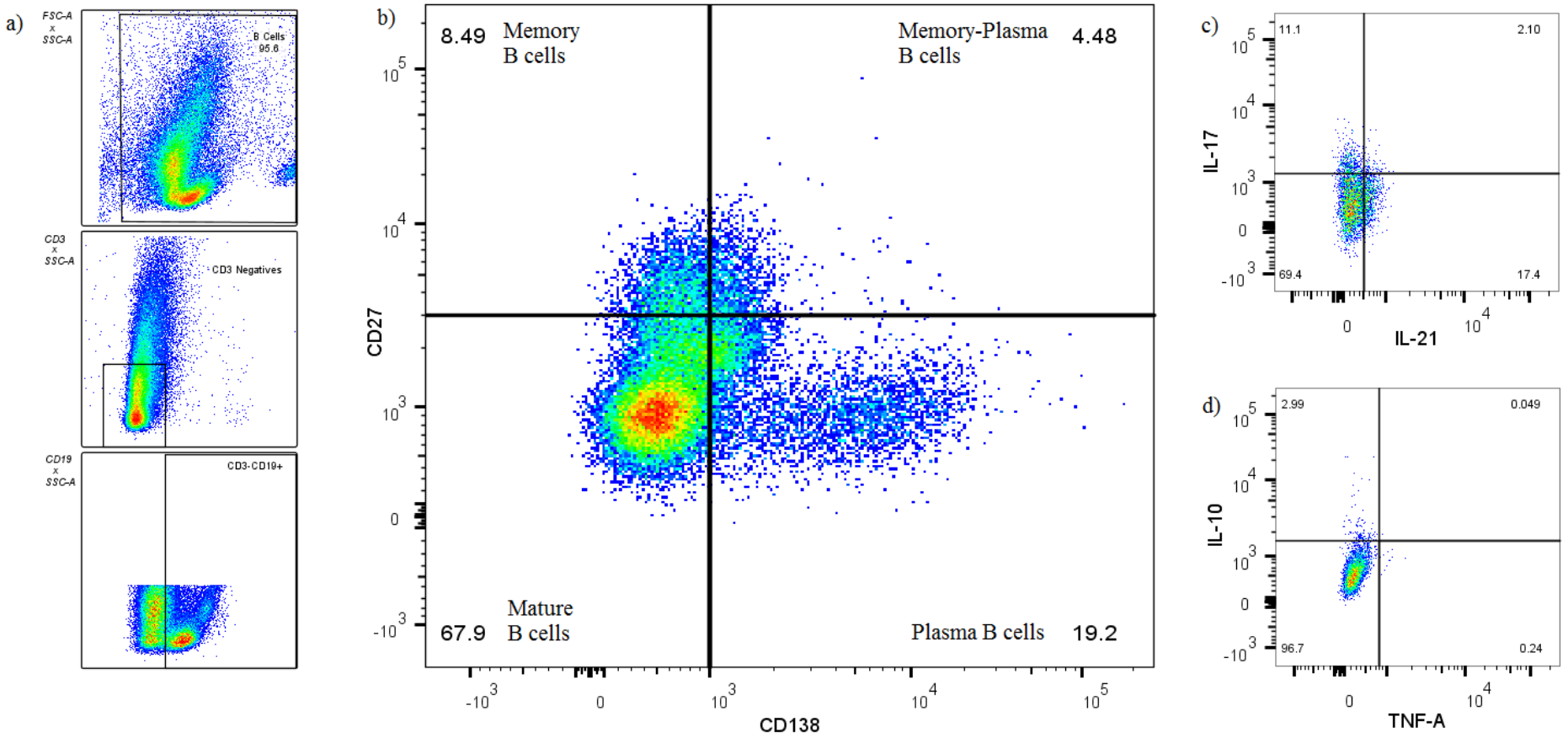
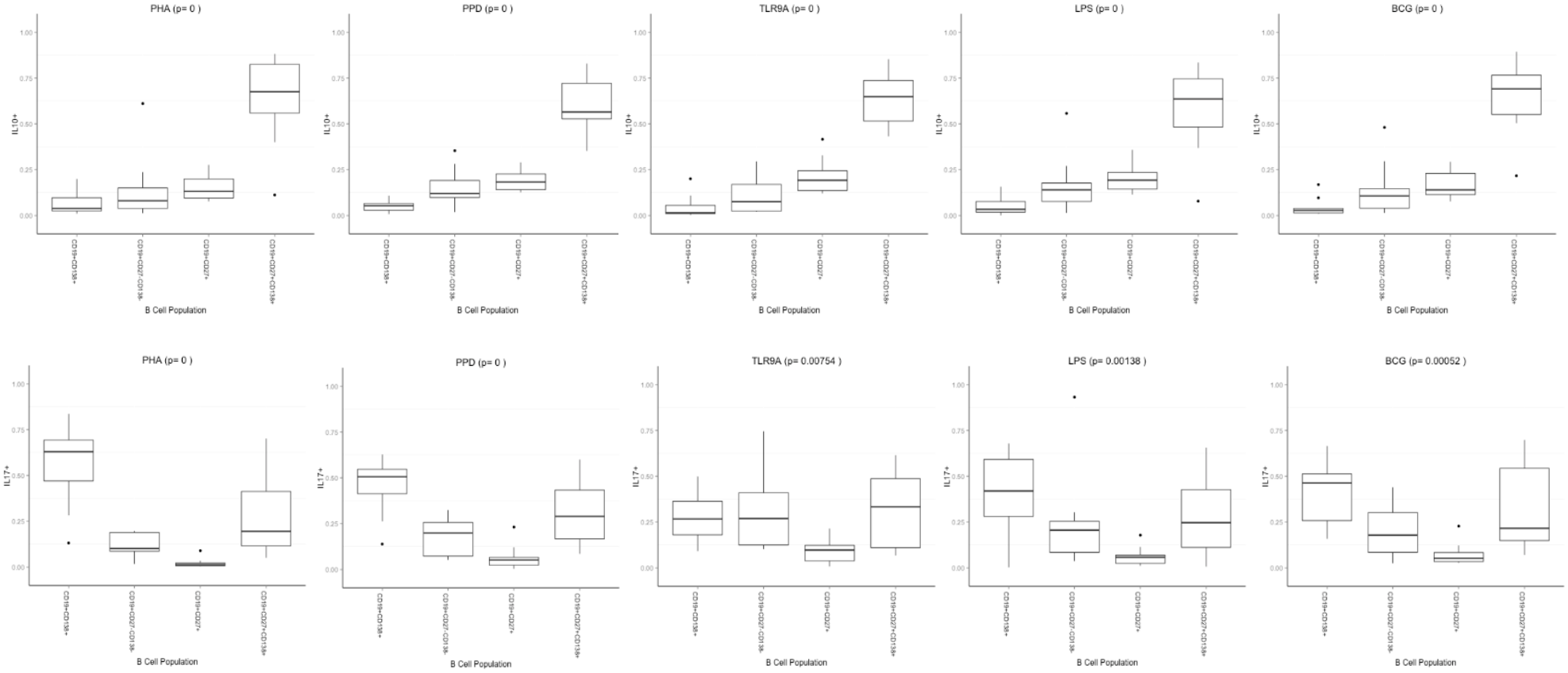


Figure 4. B cell flow cytometry gating strategy. a) Initial cell- inclusion and exclusion gating. b) The main B cell phenotypes from which cytokine production were assessed includes memory B cells ($CD19^{+}CD27^{+}$), mature B cells ($CD19^{+}CD27^{-}CD138^{-}$), plasma B cells ($CD19^{+}CD138^{+}$) and plasma-memory B cells ($CD19^{+}CD27^{+}CD138^{+}$). c) Further downstream cytokine analysis were done following the identification of the main B cell subsets in b), by plotting respective cytokines against one another. Appropriate cut-off levels for the gating strategy were used as determined by using FMO control samples.



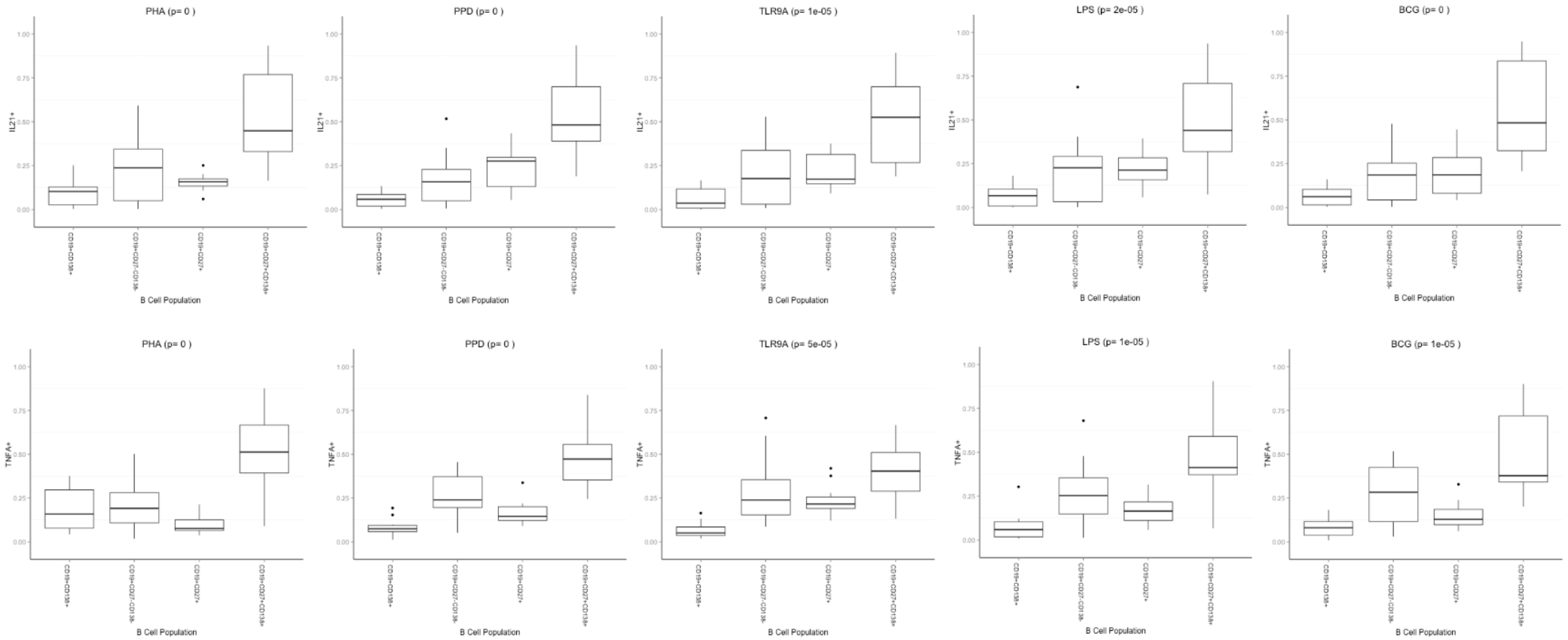


Figure 5. Plasma-memory B cells ($CD19^+CD27^+CD138^+$) are major contributors to cytokine production. B cells were stimulated for 16-hours with various antigens, whereafter flow cytometric analysis were performed to assess intracellular cytokine production of specific B cell subsets. Each box-plot represents the relative cytokine production as contributed by the respective B cell subset, and according to stimulating condition. The contribution of each B cell subset in a graph adds up to 100% of the total respective cytokine's production, for that stimulating condition. Graphs are included for the frequency of the four cytokine measured, including IL-10, IL-17, IL-21 and TNF-α.

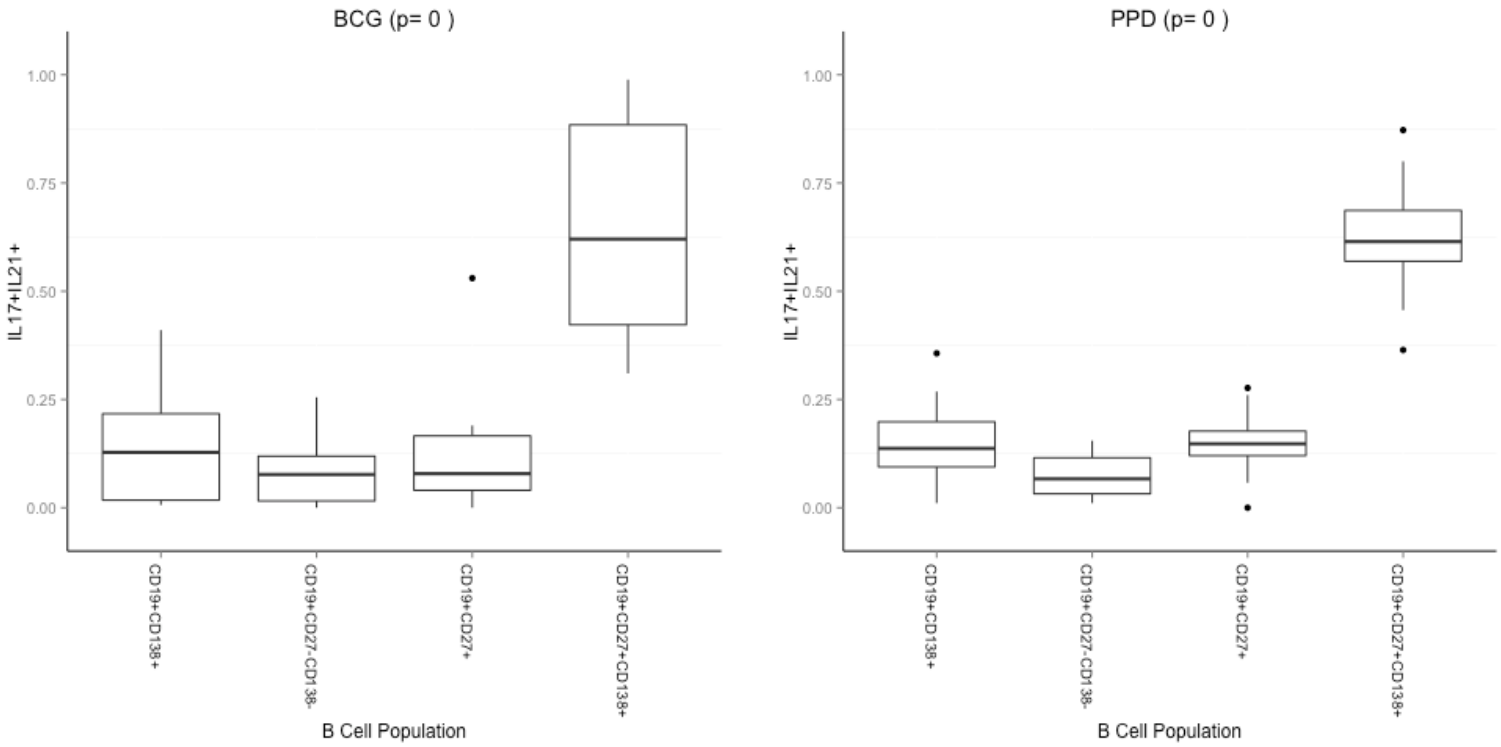


Figure 6. Plasma-memory B cells may be key B cells subsets during innate recruitment of B cells during tuberculous challenge. B cells were stimulated for 16-hours with BCG and PPD respectively. Flow cytometric analysis of various B cell subsets indicated that plasma-memory B cells were the major contributors for the dual production of IL-17 and IL-21, implicating them in the potential recruitment of innate-like B cells during *M.tb* challenge. Each box-plot represents a fraction from each B cell subset to the total cytokine production

Table 1. Top five pathways B cell derived IL-1β is implicated in.

Stimulant	Marker	Top 5 Pathways	p - Value
BCG	IL-1β	1. Role of Cytokines in Mediating Communication between Immune Cells	5,83E-11
		2. T Helper Cell Differentiation	1,65E-10
		3. Altered T Cell and B Cell Signalling in Rheumatoid Arthritis	3,58E-10
		4. Communication between Innate and Adaptive Immune Cells	3,77E-10
		5. Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses	1,71E-09

Chapter 4:

Summary and concluding remarks

In this study we hypothesised that B cells play a role during tuberculosis infection and disease, and that treatment has an influence on its frequency, maturation and role as effectors/regulators. We aimed to identify the various phenotypical B cell subsets during disease and the subsequent treatment response. Further, we wanted to elucidate the functional capacity of these cells following a broad range of antigenic challenges – to assess and quantify specific functional capacity.

From our in-depth immunophenotyping, multicytokine analysis and flow cytometry-based functional analysis experiments we learned that:

- Memory-based B cell phenotypes and marginal zone (MZ) B cells can distinguish between TB at diagnosis and end of treatment (week 24), in both class-switched (CS) and non-class switched (NCS) variants.
- Class-switched marginal zone and NCS mature B cell frequencies are distinguishable at diagnosis between tuberculosis and individuals diagnosed with other-lung diseases (OLD).
- Mature B cells best distinguish between all three groups (TB, OLD and healthy community controls) at diagnosis, in both the CS and NCS variants.
- B cells readily and differentially produce pro-inflammatory cytokines following antigenic challenge.
- BCG stimulation results in the significantly higher production of IL-1β compared to the other stimulations.
- TLR-based stimulations resulted in the greatest cytokine yields from B cells. From the Ingenuity Pathway Analysis (IPA), we learned that the production of B cell derived IL-1β primarily facilitates pathways implicated in intra-cellular communication.
- Plasma-memory B cells ($CD19^{+}CD27^{+}CD138^{+}$) are the primary contributors of cytokine (IL-10, IL-21 and TNF-α), except for IL-17 which seems to be mainly produced by plasma B cells ($CD19^{+}CD138^{+}$).

Taken together, these results confirm that tuberculosis disease results in the generation of unique B cell frequencies. It also confirms that chemotherapeutic intervention could have an impact on these frequencies, as we observed significant changes in frequencies over the course of treatment. These unique B cell frequencies during tuberculosis disease identify them as prime candidates for potential biomarkers of disease and treatment

response. Lastly, these results show that B cells readily respond to antigenic challenge in a non-humoral fashion.

Project limitations:

- A limited B cell panel for the immuno-phenotyping. IgD is a good marker to exclude doubt with respect to CS and NCS (IgM vs IgD phenotypes). Another possible antibody for inclusion would be CD21 as this would give further insight into the phenotypes activation state. CD21 is also present on a broad range of B cells from transitional 2B B cells through to activated germinal centre B cells which would increase the accuracy of identified subsets. Other antibodies to consider would include CD5, CD45R and CD1d (specifically for MZ B cells).
- Study sample numbers. A total of 11 samples were collected for the functional component of the project. Ideally, this number would be increased to at least 20 to have statistical power.
- TB disease group. We did not include a group with active TB disease in the functional analysis of B cells. As almost 70ml of blood was required per participant to meet the minimum required B cell counts for the functional work, the acquisition of these samples were not possible within the time constraints of this project. Similarly, a third group, namely individuals diagnosed with other-lung diseases would've strengthened the analysis.

Future research perspectives:

- A confirmatory study with an expanded B cell immunophenotyping panel to not only more accurately define phenotypes, but to also confirm unique frequencies and strengthen the use of these B cell phenotype frequencies as potential biomarkers.
- A site of disease research study with the inclusion of broncho-alveolar lavage (BAL) samples for functional B cell responses and immunophenotyping. This research would give more accurate insight into the B cell derived immune response from the active TB-disease group
- Further analysis should include the transcriptomic analysis of B cells and potentially measure the epigenetic alterations.
- B cell miRNA should also be measured to give specific insight into B cell transcriptomics during *M.tb* challenge. Potential miRNA to study which are B cell specific include:
    - miR-101-3p
    - miR-204-5p
    - miR-331-3p
    - miR-210
    - miR-145-5p

    - miR-34a-5p
- The measurement of anti-*M.tb* antibodies and a correlation with specific B cell subset frequencies or B cell-specific cytokine production could aid in the monitoring of disease progression or response to TB treatment.

Conclusion

This project highlighted unique B cell phenotypes and frequencies during tuberculosis disease which hold promise as biomarkers of disease and treatment response evaluation. New insight into specific B cell host-pathogen responses following a broad range of antigenic challenges were gained, which confirmed their role as potential mediators of immunity in an effector or regulatory role. These results pave the way for further research into the extent at which B cells participate in host immune responses. Further emphasis should be placed on identifying the specific phenotype of B cells under conditions of anti-TB treatment, treatment failure and relapse with regards to identify the function beneficially to resolving the *M.tb* infection and those cells which contribute to immunopathology. The identification of these subsets could set the scene for targeted drug development and tailored treatment plans for tuberculosis.

Appendix

A1. MSD Protocol

MSD® Proinflammatory Panel 1 (human) Control Pack

Ordering Information

MSD Customer Service
Phone: 1-240-314-2795
Fax: 1-301-990-2776
Email: CustomerService@mesoscale.com
www.mesoscale.com/support

Scientific Support

Phone: 1-240-314-2798
Email: ScientificSupport@mesoscale.com
www.mesoscale.com/support

Company Address

MESO SCALE DISCOVERY®
A division of
Meso Scale Diagnostics, LLC.
1601 Research Boulevard
Rockville, MD 20850-3173 USA

www.mesoscale.com®

For Research Use Only.
Not for use in diagnostic procedures.

Catalog #	C4049-1
Contents	Proinflammatory Panel 1 (human) Control 1 (5 vials,* lyophilized) Proinflammatory Panel 1 (human) Control 2 (5 vials,* lyophilized) Proinflammatory Panel 1 (human) Control 3 (5 vials,* lyophilized) *If supplied as part of a V-PLEX® Plus kit, quantity will match number of plates ordered.
Storage	2–8°C

Summary and Intended Use

The use of controls to monitor the analytical performance and reliability of test methods is an essential component of good laboratory practice.

The Proinflammatory Panel 1 (human) Control Pack consists of 3 levels of controls, each containing known concentrations of all human cytokines that are detected by the Proinflammatory Panel 1 (human) Kit. Proinflammatory Panel 1 (human) Controls 1, 2, and 3 are prepared by spiking recombinant calibrators into a non-human serum matrix. The controls are supplied as lyophilized powder.

Storage and Handling

To maximize the consistency of measured values across vials, the controls must be stored at the temperature recommended above. Once reconstituted in 250 µL of Diluent 2, the controls can be stored up to 10 days at 2-8°C. For long term storage, reconstituted controls must be stored frozen at ≤-70°C. Reconstituted controls can go through 3 freeze–thaw cycles without significantly changing analytes levels. Discard unused reconstituted material after the third freeze–thaw cycle.

To use, first reconstitute each vial of Proinflammatory Panel 1 (human) controls in 250 µL of Diluent 2, and then dilute to match the sample dilution (2-fold is the recommended sample dilution for this kit). Add diluted control solutions directly to the MSD Cytokine Panel 1 (human) plate, and assay as unknown samples. Discard excess diluted control material after use.

Safety

Use safe laboratory practices and wear gloves, safety glasses, and lab coats when handling controls. Handle and dispose of all hazardous samples properly in accordance with local, state, and federal guidelines. Additional product-specific safety information is available in the safety data sheet, which can be obtained from MSD Customer Service.

Assignment of Control Values

The controls are provided to assess reproducibility of assay performance, and precision CVs are expected to be less than 25%. The certificate of analysis contains the concentrations of the controls measured at MSD across three lots. Even with good laboratory practices, site-to-site differences may occur; therefore, to establish accuracy specifications, it is recommended that each lab should establish its own nominal values and acceptance range for the controls concentrations.

Spot the Difference®

MSD®

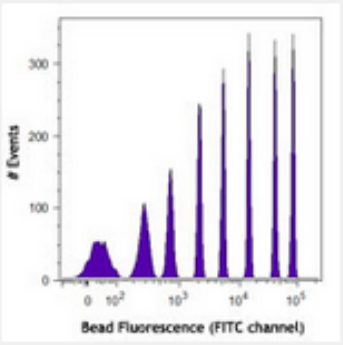
MK-DS-200-k3-2014May

A2. Flow Controls

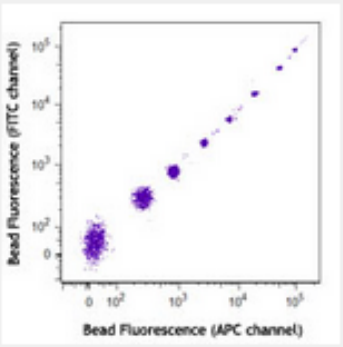
A2.1 Rainbow Beads

Product Details

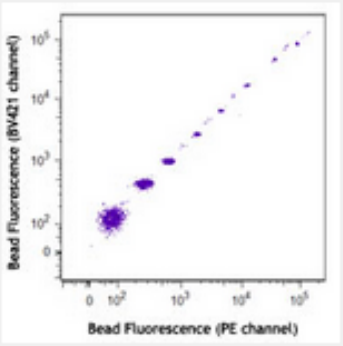
Formulation:	Rainbow Calibration Particles are in deionized water with 0.02% sodium azide and 0.01% NP-40.
Concentration:	10^7 particles per ml.
Storage & Handling:	Store between 2°C and 8°C. Do not freeze. Protect from light.
Application:	FC
Recommended Usage:	Resuspend well (e.g. vortex briefly) before adding 3-5 drops to 1 ml of filtered DI water.
Application Notes:	Use of Rainbow Calibration Particles, 8 peaks (3.0-3.4 µm), is common for the following purposes: 1. Instrument performance verification (linearity) and monitoring 2. Standardization of instrument settings for longitudinal studies (can also use Rainbow Calibration Particles, 6 peaks (Cat. No. 422901), and single-peak Rainbow Fluorescent Particles (Cat No. 422905 and 422907) 3. Evaluation of new collection optics (e.g. bandpass filters) The relative number of fluorophores per particles has been determined for every peak of this product in FL1 (FITC and MEFL), FL2 (RPE and MEPE), FL3 (RPECy5 and MEPCY) and FL4 (APC and MEAP) channels on a flow cytometer to plot the calibration graph, which can be used to check linearity of the PMT in each channel. PMT QC templates can be downloaded directly from Spherotech™.
Application References:	1. Calibration and Performance Tracking of Flow Cytometers Using Sphero™ Calibration Particles 2. Measuring Molecules of Equivalent Fluorochrome (MEF) Using Sphero™ Rainbow and Ultra Rainbow Calibration Particles 3. Determining PMT Linearity in Flow Cytometers Using the Sphero™ PMT Quality Control Excel Template 4. Perfetto SP, *et al.* 2006. *Nat. Protoc.* 1:1522. Publication Library



Bead fluorescence at 488 nm excitation with a 530/30 BP filter (FITC channel).



Bead fluorescence at 640 nm excitation with a 670/30 BP filter (APC channel) plotted against bead fluorescence at 488 nm excitation with a 530/30 BP filter (FITC channel).



Bead fluorescence at 561 nm excitation with a 582/15 BP filter (PE channel) plotted against bead fluorescence at 405 nm excitation with a 450/50 BP filter (BV421™ channel).

A2.2 Compensation Beads

BD™ CompBeads

Technical Data Sheet

Anti-Mouse Ig, κ/Negative Control Compensation Particles Set

Product Information

Material Number:	**552843**
Component:	**51-90-9001229**
Description:	Anti-Mouse Ig, κ
Size:	6.0 ml (1 ea)
Storage Buffer:	Aqueous buffered solution containing BSA and ≤0.09% sodium azide.
Component:	**51-90-9001291**
Description:	Negative Control
Size:	6.0 ml (1 ea)
Storage Buffer:	Aqueous buffered solution containing BSA and ≤0.09% sodium azide.

Description

The BD™ CompBeads Set Anti-Mouse Ig, κ are polystyrene microparticles which are used to optimize fluorescence compensation settings for multicolor flow cytometric analyses. The set provides two populations of microparticles, the BD™ CompBeads Anti-Mouse Ig, κ particles, which bind any mouse κ light chain-bearing immunoglobulin, and the BD™ CompBeads Negative Control, which has no binding capacity. When mixed together with a fluorochrome-conjugated mouse antibody, the BD™ CompBeads provide distinct positive and negative (background fluorescence) stained populations which can be used to set compensation levels manually or using instrument set-up software. Since the compensation adjustments are made using the same fluorochrome-labeled antibody to be used in the experiment, this method allows the investigator to more accurately establish compensation corrections for spectral overlap for any combination of fluorochrome-labeled antibodies (without having to use valuable tissue samples or hard-dyed beads with potentially mismatched fluorescence spectra). Use of the BD™ CompBeads is highly recommended for use in all experiments using tandem dye (i.e., PE-Cy™7, APC-Cy™7, etc.) conjugates, which may have distinct spectral characteristics for each conjugate.

Note: BD Horizon™ V500 and AmCyan conjugated reagents can show significant differences in emission spectrum on stained cells and when captured on BD™ CompBeads. Thus, spillover values for these dyes evaluated with BD™ CompBeads may not provide correct compensation for cells. Therefore, single stained cellular controls are recommended to set up compensation for AmCyan and BD Horizon™ V500 reagents. BD Horizon™ V500-C has been modified to minimize these spectral differences and BD™ CompBeads may be used to determine spillover values for RUO antibodies conjugated to BD Horizon™ V500-C.

Preparation and Storage

Store undiluted at 4°C and protected from prolonged exposure to light. Do not freeze.

Application Notes

Application

Flow cytometry	Routinely Tested

Recommended Assay Procedure:

This BD™ CompBeads Set has been tested with mouse Ig antibodies conjugated to various fluorochromes and analyzed using a BD FACS brand flow cytometer to ensure specificity and reactivity of the particles. See the specific instructions below on the use of the BD™ CompBeads Set:

1. Vortex BD™ CompBeads thoroughly before use.
2. Label a separate 12 x 75 mm sample tube (BD Falcon™, Cat. No. 352008) for each flurochrome-conjugated mouse Ig, κ antibody to be used on a given experiment.
3. Add 100 µl of staining buffer [e.g., BD Pharmingen Stain (FBS), Cat. No. 554656 or BD Pharmingen Stain (BSA), Cat. No. 554657] to each tube.
4. Add 1 full drop (approximately 60 µl) of the BD™ CompBeads Negative Control and 1 drop of the BD™ CompBeads Anti-Mouse Ig, κ beads to each tube and vortex.
5. Add 20 µl of each prediluted antibody stock (diluted to a concentration optimal for staining 10^6 cells) to be tested on a given experiment to the appropriately-labeled tube. (Make sure the antibody is deposited to the bead mixture, then vortex.)
6. Incubate 15 - 30 minutes at room temperature. Protect from exposure to direct light.
7. During the incubation of beads and antibody, set the flow cytometer instrument PMT voltage settings using the target tissue for the given experiment (eg, whole blood, splenocytes, etc). If you are unsure, use the BD™ CompBeads Negative Control beads as your negative reference point and proceed.
8. Following the incubation step (see Step 6 above), add 2 ml staining buffer to each tube and pellet by centrifugation at 200 x g for 10 minutes.

BD Biosciences

bdbiosciences.com

United States	Canada	Europe	Japan	Asia Pacific	Latin America/Caribbean
877.232.8995	800.979.9408	32.53.720.550	0120.8555.90	65.6861.0633	55.11.5185.9995

For country contact information, visit bdbiosciences.com/contact

9. Discard supernatant from each tube by careful vacuum aspiration using a fine-tip Pasteur pipette.
10. Resuspend bead pellet in each tube by adding 0.5 ml of staining buffer to each tube. Vortex thoroughly.
11. Run each tube separately on the flow cytometer. Gate on the singlet bead population based on FSC (forward-light scatter) and SSC (side-light scatter) characteristics.
12. Adjust flow rate to 200 - 300 events per second if possible.
13. Create a dot plot for the given fluorochrome-conjugated antibody as appropriate [i.e., to set compensation for a fluorescein (FITC)-conjugated antibody, use an FL1 vs. FL2 dot plot].
14. Place a quadrant gate such that the negative bead population is in the lower left quadrant and the positive bead population is in the upper or lower right quadrant, and adjust the compensation values until the median fluorescence intensity (MFI) of each population (as shown in the quadrant stats window) is approximately equal (i.e., for FL2 -%FL1, the FL2 MFI of both bead populations should be approximately equal when properly compensated).
15. Repeat Steps 13 and 14 for other tubes, as necessary.
16. Proceed to acquiring the actual staining experiment.

Product Notices

1. Since applications vary, each investigator should titrate the reagent to obtain optimal results.
2. Cy is a trademark of Amersham Biosciences Limited.
3. Source of all serum proteins is from USDA inspected abattoirs located in the United States.
4. Caution: Sodium azide yields highly toxic hydrazoic acid under acidic conditions. Dilute azide compounds in running water before discarding to avoid accumulation of potentially explosive deposits in plumbing.
5. Please refer to www.bdbiosciences.com/pharmingen/protocols for technical protocols.

BD Biosciences
bdbiosciences.com

United States	Canada	Europe	Japan	Asia Pacific	Latin America/Caribbean
877.232.8995	800.979.9408	32.53.720.550	0120.8555.90	65.6861.0633	55.11.5185.9995

For country contact information, visit bdbiosciences.com/contact

A3. R-Script

```
# Set directory and read in data

setwd("/Volumes/Seagate Backup Plus Drive/projects/duPlessis")

library(ggplot2)

library(foreign)

jac=read.csv("jac4.csv")

cstr = c("CD19+CD27+","CD19+CD138+","CD19+CD27-CD138-","CD19+CD27+CD138+")

tempcell=toupper(jac$Phenotype)

celltype=NULL

pheno=NULL

for (k in 1:length(cstr)){

  for (i in 1:length(tempcell)){

    if (cstr[k]==substr(tempcell[i],1,nchar(cstr[k]))){

      celltype[i]=substr(tempcell[i],nchar(cstr[k])+1,nchar(as.vector(tempcell[i])))

      pheno[i]=cstr[k]

    }

  }

}

jac.dat=data.frame(jac,celltype,pheno)


# Summarize data into means and confidence length

ci <-function(x){1.96*sd(x)/sqrt(length(x))}

mean.dat=aggregate(jac.dat$percent,
by=list(jac.dat$Stimulant,jac.dat$celltype,jac.dat$pheno),FUN="mean")

ci.dat=aggregate(jac.dat$percent, by=list(jac.dat$Stimulant,jac.dat$celltype,jac.dat$pheno),FUN="ci")

names(mean.dat)<-c("stimulant","celltype","phenotype","meanpercent")
```

```
names(ci.dat)<-c("stimulant","celltype","phenotype","cipercent")

agg.dat=merge(mean.dat,ci.dat,by=c("stimulant","celltype","phenotype"))


# Editing the phenotype labels - taking off the cell type attached to phenotype

#stoppos = -nchar(as.vector(agg.dat$celltype))+nchar(as.vector(agg.dat$phenotype))

#pheno = substr(agg.dat$phenotype,1,stoppos)

#agg.dat=data.frame(agg.dat,pheno)


# Individuals graphs for each stimulant and cell type & function

# Compares phenotypes


st = levels(agg.dat$stimulant)

ct = levels(agg.dat$celltype)


for (i in 1:length(st)){

  for (j in 1:length(ct)) {

    pv=anova(lm(percent~as.factor(pheno),data=subset(jac.dat,Stimulant==st[i] & celltype==ct[j])))$"Pr(>F)"[1]

    q=ggplot(subset(agg.dat,stimulant==st[i] & celltype==ct[j]), aes(x=phenotype,y=meanpercent))+geom_bar(stat="identity",fill="lightgrey")+

    ggtitle(paste(st[i],"(p=",round(pv,5),")"))+ylab(ct[j])+xlab("B Cell Population")+coord_cartesian(ylim = c(-0.1,1.1))+

    theme(panel.background=element_rect(fill = "transparent"), axis.line = element_line(colour = "black"))+

    geom_errorbar(aes(ymin=meanpercent-cipercent, ymax=meanpercent+cipercent), width=.2, colour="red")+geom_point(colour="red")

    q=q+theme(axis.text.x = element_text(angle = -90, hjust = 0,colour="black"))

    ct1=gsub("[[:punct:]]", "",ct[j])

    ct1=gsub(" ","",ct1)
```

```
    outputfile = paste(st[i],"_",ct1,".png",sep="")
    png(outputfile)
    print(q)
    dev.off()
}
}

# Boxplots
st = levels(jac.dat$Stimulant)
ct = levels(jac.dat$celltype)

for (i in 1:length(st)){
  for (j in 1:length(ct)) {
    pv=anova(lm(percent~as.factor(pheno),data=subset(jac.dat,Stimulant==st[i] & celltype==ct[j])))$"Pr(>F)"[1]
    q=ggplot(subset(jac.dat,Stimulant==st[i] & celltype==ct[j]), aes(x=pheno,y=percent))+
      ggtitle(paste(st[i],"(p=",round(pv,5),")"))+ylab(ct[j])+xlab("B Cell Population")+coord_cartesian(ylim = c(-0.1,1.1))+
      theme(panel.background=element_rect(fill = "transparent"), axis.line = element_line(colour = "black"))+
      geom_boxplot()
    q=q+theme(axis.text.x = element_text(angle = -90, hjust = 0,colour="black"))
    ct1=gsub("[[:punct:]]", "",ct[j])
    ct1=gsub(" ","",ct1)
    outputfile = paste("box","-",st[i],"_",ct1,".png",sep="")
    png(outputfile)
    print(q)
    dev.off()
```

```
  }

}
```